

# **Utility of the precision cut lung slice model to investigate airway smooth muscle contraction**

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## **ABSTRACT**

Asthma is characterised by airway remodelling and an increase in airway resistance. A greater understanding of the mechanisms involved in airway inflammation and airway hyper-responsiveness (AHR) may highlight therapeutic opportunities for asthma. This study initially aimed to optimise the preparation of precision cut lung slices (PCLS) in mouse and pig to investigate the influence of calcium ( $\text{Ca}^{2+}$ ) homeostasis on airway smooth muscle (ASM) contraction as a prelude to human studies. The PCLS technique was then applied to a murine model of allergic airway disease to explore the inflammatory process and pathogenesis of airway hyper-reactivity in sensitised mice.

Initial experiments using murine and porcine airways validated the PCLS model and demonstrated the significance of release and refilling of  $\text{Ca}^{2+}$  from internal stores to induce and maintain an airway contraction. Results also highlight interesting species differences in agonist sensitivity, with the porcine system sharing similar pharmacology to human airways.

Using a murine model of allergic airway disease, agonist induced contractile responses in peripheral airways were measured *in vitro* using the PCLS technique. BALB/c mice underwent initial sensitisation by intraperitoneal administration of ovalbumin, receiving a 3 day challenge with aerosolised OVA 1% (v/v), for varying periods of up to 3 weeks for acute, mid-chronic and chronic sensitisation protocols. To investigate the influence of the inflammatory environment, naïve murine lung slices were incubated with selected inflammatory mediators. OVA sensitisation led to progressive structural remodelling and AHR to methacholine (MCh) challenge. However, this hyper-responsiveness was decreased 48 hours post lung removal. Of the inflammatory mediators selected for lung slice incubation, IL-33 significantly increased AHR to MCh. IL-33 is a proinflammatory cytokine with transcriptional repressor properties, playing a role in initiating the  $\text{T}_\text{H}2$  inflammatory response. In lung slices prepared from IL-33 receptor (ST2) KO mice IL-33 was unable to sensitise the contractile response. These data suggest the inflammatory environment promotes AHR and disassociates this airway sensitivity from structural remodelling. These data suggest a key role for IL-33 in mediating AHR in this murine model.

Investigation of the mechanisms involved in airway hyper-reactivity revealed mRNA expression of IL-33 and the IL-33 receptor (ST2) in soluble and membrane bound forms were



significantly increased in the mid-chronic and chronic ovalbumin sensitised murine lung tissue. Further quantitative analysis in human lung showed expression of IL-33 in epithelial and ASM cells. The human ST2 receptor (also known as IL-1RL-1) was expressed in mast cells. Together these results suggest IL-33 is a sensor of tissue damage; indirectly inducing AHR through further inflammatory cell activation to target ASM. This study demonstrates IL-33's role as an inflammatory marker of asthma and suggests a novel therapeutic intervention by targeting of the ST2 receptor.

## **ABSTRACTS & ORAL COMMUNICATIONS**

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## Abbreviations

$\Delta$	Delta
2-APB	2-Aminoethoxydiphenyl borate
AHR	Airway hyper-responsiveness
5-HT	Serotonin
ACh	Acetylcholine
Ab	Antibody
APC	Antigen presenting cells
ASM	Airway smooth muscle
ATP	Adenosine triphosphate
BAPTA	1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid
BLAST	Basic Local Alignment Search Tool
BM	Basement membrane
cAMP	Cyclic adenosine-3',5'-monophosphate
CCL	Chemokine ligand
CIF	Calcium influx factor
CRAC	Ca <sup>2+</sup> release activated Ca <sup>2+</sup> channels
COX-2	Cyclooxygenase-2
CPA	Cyclopiazonic acid
CT	Computerised tomography
DAG	Diacylglycerol
DC	Dendritic cells
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
EGTA	Ethylene glycol tetraacetic acid
ECM	Extra cellular matrix
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
FCS	Foetal calf serum
GATA3	GATA 3 binding protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gd <sup>3+</sup>	Gadolinium ions
GDP	Guanisine diphosphate
GM-CSF	Granulocyte macrophage-colony stimulating factor
GRE	Glucocorticoid response element
GTP	Guanisine triphosphate
GPCR	G protein coupled receptor
H <sub>2</sub> O	Water
HA	Histamine
HASM	Human airway smooth muscle
HBEC	Human bronchial epithelial cells
HBSS	Hank's buffered saline solution
HEPES	N-2-Hydroxyethylpiperazine-N-2-Ethanesulfonic Acid
HIS	Histamine
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
ICAM	Intercellular adhesion molecule
I <sub>K</sub> B $\alpha$	Inhibitor of nuclear factor- $\kappa$ B
IFN- $\gamma$	Interferon- $\gamma$
IL	Interleukin
IL-1RL-1	Interleukin 1 receptor like 1
IP <sub>3</sub>	Inositol triphosphate
IP <sub>3</sub> R	Inositol triphosphate receptor
KO	Knock out
LABA	Long acting $\beta_2$ -adrenoreceptor agonist
La <sup>3+</sup>	Lanthanum ions
M3 R	Muscarinic acetylcholine Receptor type 3
MAPK	Mitogen activated protein kinase
MCh	Methacholine
MgCl <sub>2</sub>	Magnesium chloride

MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
mRNA	Messenger ribonucleic acid
NF- $\kappa$ B	Nuclear factor-K B
Ni <sup>2+</sup>	Nickel ions
PBS	Phosphate buffer Saline
PDGF	Platelet derived growth factor
PI	Phosphoinositide
PIP <sub>2</sub>	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PLC- $\beta$	Phospholipase C
Q-PCR	Quantitative PCR
RANTES	Regulated on activation, normal T cell expressed
rpm	Revolution per minute
RNA	Ribonucleic acid
ROCC	Receptor operated calcium channel
SABA	Short acting $\beta_2$ -adrenoreceptor agonist
SCF	Stem cell factor
SERCA	Sarcoplasmic/endoplasmic reticulum Ca <sup>2+</sup> -ATPase
siRNA	Small interfering RNA
SOCC	Store operated calcium channels
STIM	Stromal Interaction Molecule
TGF- $\beta$	Transforming growth factor - $\beta$
TRP	Transient receptor potential
TNF- $\alpha$	Tumour necrosis factor- $\alpha$
TSLP	Thymic stromal lymphopoietin
VEGF	Vascular endothelial growth factor
VCAM	Vascular adhesion molecule
VOCC	Voltage operated calcium channel



# **CHAPTER ONE**

## **INTRODUCTION**

## 1. CHAPTER ONE

### 1.1 ASTHMA

Asthma is classed as a chronic inflammatory disorder of the airways (Homer and Elias 2005) and is estimated to affect approximately 10% of the population during their life span (Fuhlbrigge, Adams et al. 2002). In some patients, chronic inflammation can lead to structural changes, referred to as airway remodelling; characterised by an increase in extracellular matrix deposition, an increase in airway wall thickness and an increase in smooth muscle mass (Elias, Zhu et al. 1999; Homer and Elias 2000; Vignola, Mirabella et al. 2003). Lung inflammation and remodelling contribute to the development of such characteristic asthmatic features as airway obstruction and airway hyper-responsiveness (AHR) (Pare, Bai et al. 1997; Barnes 1998; Busse, Banks-Schlegel et al. 2004). Asthma is a complex, heterogeneous disease caused by the interaction of multiple environmental factors with genetic factors with greater than 100 major and minor susceptibility genes implicated (Umetsu, McIntire et al. 2002; von Mutius 2009). Several different phenotypes including allergic asthma (most common) (Robinson, Hamid et al. 1992), exercise induced (Carlsen and Carlsen 2002) and occupational asthma (Lombardo and Balmes 2000) exist. Theories on the initiation of the inflammatory process in asthma suggest the origin of the disease usually occurs early in life and involves a complex interplay between genetic and environmental factors at critical times (Reed 2006).

Our understanding of the inflammatory response in asthma has changed drastically in recent years with an emphasis on the link between the innate and adaptive human immune system (Turvey and Broide 2011). Investigation of the development and regulation of asthma has focused on the balance between  $T_H1$  and

$T_H2$  cytokine induced pathways with a shift towards a  $T_H2$  profile (Barnes 2008) being present in most patients. The epithelial cell produced cytokines, thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 may be the link between the innate and adaptive immune system to drive this  $T_H2$  response (Bulek, Swaidani et al. 2011; Coffman 2011; Paul and Zhu 2011; Zhu, Yamane et al. 2011; Barrett and Austen 2009).

### 1.1.1 The innate response

Innate immunity is present in all multi cellular organisms, while adaptive immunity evolved much later in jawed fish and all succeeding vertebrates (Pancer and Cooper 2006; Rolff 2007). Innate immune protection involves such hematopoietic cells as mast cells, dendritic cells, macrophages, neutrophils and eosinophils (Iwasaki and Medzhitov 2011; Turvey and Broide 2011; Barrett and Austen 2009). Skin and epithelial cells lining the respiratory and gastrointestinal tract contribute. The innate immune response involves a limited number of genetically predetermined germline encoded receptors (such as toll like receptors (TLR)) that recognise a variety of pathogen associated molecular patterns (PAMP); these are highly conserved structures expressed by large groups of microbes (Turvey and Broide 2011; Janeway and Medzhitov 2002). The receptors enable the innate defense system to discriminate pathogens and determine the appropriate cascade for adaptive responses (Turvey and Broide 2011). Host defense appears to be achieved through interface of innate and adaptive immunity; with contribution from the epithelial cell produced cytokines TSLP, IL-25 and IL-33 (Iwasaki and Medzhitov 2011; Saenz, 2008; Barrett and Austen 2009).

TSLP is an IL-7 like cytokine (Sims, Williams et al. 2000). During allergic inflammation bronchial epithelial cells produce TSLP, which is fundamental for T<sub>H</sub>2 differentiation. TSLP is highly expressed in the epithelium of patients with asthma (Soumelis, Reche et al. 2002). TSLP acts on dendritic cells (DC) through an OX40 ligand (Arestides, He et al. 2002; Ito, Wang et al. 2005) leading to differentiation of naïve CD4<sup>+</sup> T cells to T<sub>H</sub>2 cells (Eisenbarth, Piggott et al. 2002; Soumelis, Reche et al. 2002; Ying, O'Connor et al. 2005); resulting in production of T<sub>H</sub>2 cytokines (TNF- $\alpha$ , IL-4, IL-5 and IL-13) and chemokines (CCL17, CCL22, CXCL18, CCL24) which attract neutrophils and eosinophils (Soumelis, Reche et al. 2002). A neutralising antibody for OX40L results in complete loss of ability to generate T<sub>H</sub>2 differentiation (Ito, Wang et al. 2005).

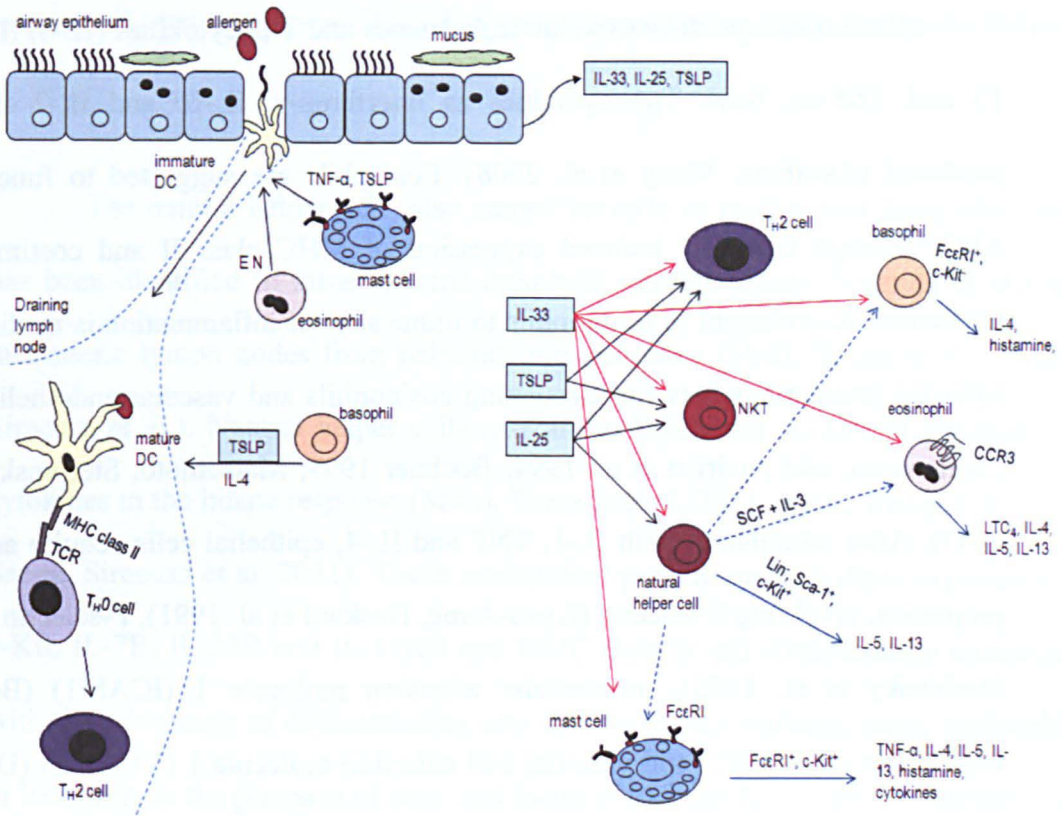
IL-25 (IL-17E) is a member of the IL-17 cytokine family (Hurst, Muchamuel et al. 2002). Of the family members, IL-25 is exceptional in promoting the T<sub>H</sub>2 response (Dong 2008). IL-25 is produced by epithelial cells in response to allergens (Saenz, Taylor et al. 2008; Hammad, Chieppa et al. 2009), activated eosinophils, bone marrow derived mast cells and basophils after Fc $\epsilon$ RI mediated activation (Barrett and Austen 2009) and also by T<sub>H</sub>2 cells (Fort, Cheung et al. 2001). Therefore IL-25 is produced in innate and adaptive immune responses. IL-25 promotes the differentiation of murine T<sub>H</sub>2 cells via an IL-4 and STAT6-dependent pathway (Angkasekwinai, Park et al. 2007). In mice exogenous administration (Fort, Cheung et al. 2001; Hurst, Muchamuel et al. 2002) of IL-25 or transgenic expression (Pan, French et al. 2001; Kim, Manoukian et al. 2002) induces asthma like features. Mouse models of OVA sensitisation have shown up regulation of the IL-25 transcript in the lung and eosinophils, CD4<sup>+</sup> T cells and IL-5 and IL-13 protein in the bronchoalveolar lavage (BAL); all of which are reduced by treatment with a

soluble IL-25 receptor prior to OVA challenge (Tamachi, Maezawa et al. 2006). Human T<sub>H</sub>2 central memory cells up regulate the IL-25 receptor on activation from TSLP stimulated DC, or from specific antigen; resulting in increased sensitivity to IL-25 and correlated with sustained expression of GATA3 (discussed below) (Wang, Angkasekwinai et al. 2007); suggesting that IL-25 may contribute to maintaining the inflammatory response.

There has been a recent explosion of evidence from genome wide association studies and sensitised animal models to suggest that IL-33 plays a fundamental role in the allergic inflammatory response. Results Chapters Four and Five of this thesis aimed to investigate the role of IL-33 in the inflammatory response and airway hyper-responsiveness. Initially IL-33 was discovered in lymph node associated endothelial cells (Baekkevold, Roussigne et al. 2003), showing the ability to relocate to the nucleus. IL-33 is now known as an IL-1 family member and has been named as the ligand for the ST2 receptor (Schmitz, Owyang et al. 2005). IL-33 mRNA expression has been found in epithelial cells, smooth muscle, fibroblasts, cardiomyocytes, keratinocytes, adipocytes and mucosal epithelial cells (Schmitz, Owyang et al. 2005; Moussion, Ortega et al. 2008; Wood, Wang et al. 2009); mainly in organs such as the skin, intestine, lung, brain and spinal cord (Hudson, Christophi et al. 2008; Moussion, Ortega et al. 2008). IL-33 protein expression in these tissues is predominantly localised to the nucleus (Baekkevold, Roussigne et al. 2003; Carriere, Roussel et al. 2007; Moussion, Ortega et al. 2008). In a similar fashion to IL-1 $\alpha$  and HMGB1 which show both nuclear and extracellular activity, it is suggested that IL-33 may have a dual function as an ‘alarmin’, with the ability to signal tissue damage to local immune cells (Moussion, Ortega et al. 2008; Cayrol and Girard 2009; Haraldsen, Balogh et al. 2009), discussed further in 6.2.

There is now evidence to suggest that inflammatory cells recruited to the airways such as mast cells, eosinophils and basophils may act as antigen presenting cells (APC) to initiate or amplify the  $T_H2$  response (Kim, DeKruyff et al.).

Mast cells express Fc $\epsilon$ RI (IgE receptor) and c-kit and are localised in tissue near mucosal surfaces and blood vessels. Mast cells degranulate in response to both i) innate immune signals such as Toll-like receptor agonists or the IL-33 cytokine (figure 1.1) (Silver, Margulis et al. 2010) and ii) adaptive signals such as cross linking of antigen-specific IgE bound to Fc $\epsilon$ RI (figure 1.2) (Prussin and Metcalfe 2006). Mast cells produce histamine, synthesise leukotriene C<sub>4</sub>, D<sub>4</sub>, E<sub>4</sub> and prostaglandin D<sub>2</sub>, chemokines and cytokines (IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-16, TNF- $\alpha$  and transforming growth factor- $\beta$  (TGF- $\beta$ ) (Brightling, Bradding et al. 2002; Galli, Kalesnikoff et al. 2005; Barrett and Austen 2009). Mast cells can also act as APCs through IL-3, IL-4 and granulocyte macrophage-colony stimulating factor (GM-CSF) which increase mast cell expression of major histocompatibility complex (MHC) class II for T cell proliferation (Kim, DeKruyff et al. 2011; Frandji, Oskeritzian et al. 1993). Mast cells originate from circulating stem cell factor (SCF)-dependent committed progenitor cells (Ochi, Hirani et al. 1999). Mast cell recruitment to the lung site of inflammation involves leukotriene B<sub>4</sub> (Weller, Collington et al. 2005), chemokines such as CCR3 (Ochi, Hirani et al. 1999) and the integrins vascular cell adhesion molecule 1 (VCAM1),  $\alpha 4\beta 1$  and  $\alpha 4\beta 7$  (Abonia, Hallgren et al. 2006). Recruitment to the ASM bundle involves the CXCR3/CXCL10 axis (Brightling, Ammit et al. 2005).



**Figure 1.1** *The innate immune response in allergic airway disease. Allergens stimulate epithelial cells to secrete TSLP, IL-25, IL-33. TSLP activates innate like natural killer T cells (NKT) to secrete  $T_H2$  cytokines. IL-25 induces  $T_H2$  cytokines IL-5 and IL-13 from natural helper cells in the absence of  $T_H2$  cells and also stimulates NKT to produce IL-13 which is linked with airway remodelling and airway hyper-responsiveness. IL-33 may influence mast cells, eosinophils, basophils, natural helper cells and NKT cells to secrete  $T_H2$  cytokines. Activated eosinophils release eosinophil derived neurotoxin (E N) which acts on dendritic cell (DC) toll like receptor 2 (TLR2) to mature DC. Mast cell derived TNF- $\alpha$  facilitates DC migration to the lymph node. Activated mast cells and basophils produce TSLP which up regulates OX40L on DC; to stimulate induction of  $T_H2$  cells by DC. SCF: stem cell factor; LTC<sub>4</sub>: leukotriene C; lin: lineage. Adapted from (Kim, DeKruyff et al. 2011; Saenz, Taylor et al. 2008).*

Basophils amplify immediate responses by releasing histamine containing granules and producing large quantities of IL-4. Basophils also show expression of MHC class II and costimulatory molecules; therefore studies suggest the ability to act as an APC to drive the  $T_H2$  response (Kim, Prout et al. 2010). These circulating granulocytes also express the IgE receptor FcεRI.

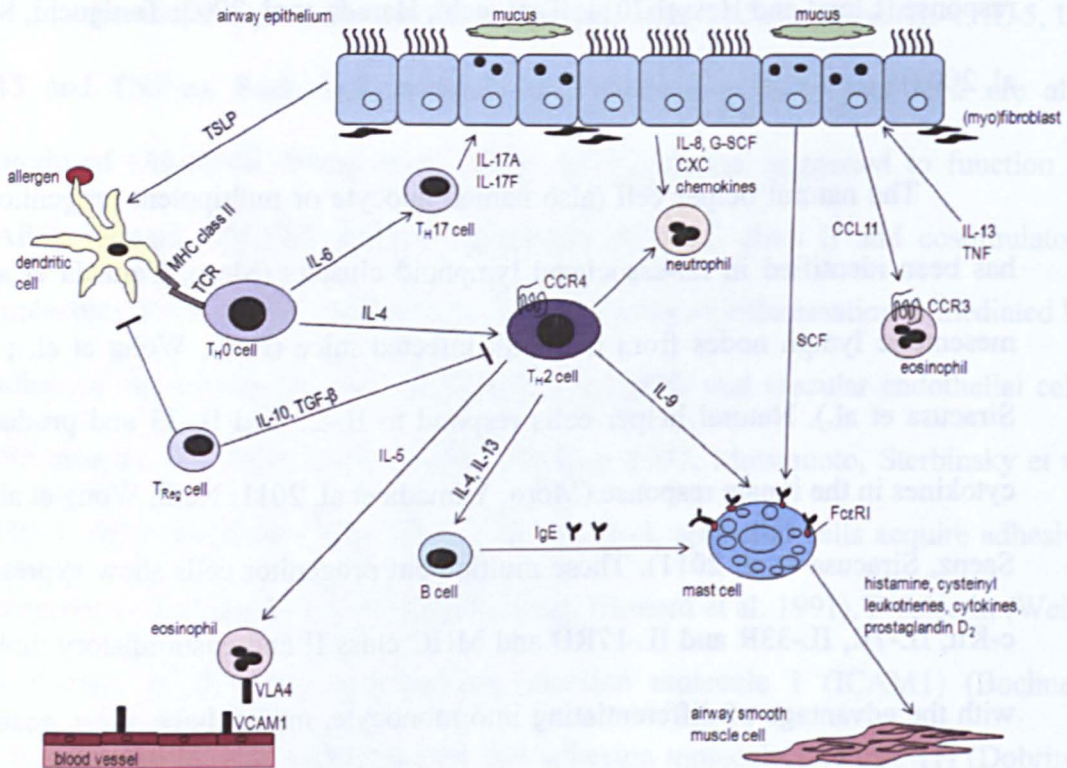
Eosinophils produce cysteine leukotrienes and  $T_H2$  cytokines (IL-4, IL-5, IL-13 and TNF- $\alpha$ ). Such  $T_H1$  cytokines as interferon- $\gamma$ , IL-10 and IL-2 are also produced (Akuthota, Wang et al. 2008). Eosinophils are suggested to function as APCs through GM-CSF induced expression of MHC class II and costimulatory molecules. Recruitment of eosinophils to tissue sites of inflammation is mediated by adhesive interactions between circulating eosinophils and vascular endothelial cells (Sriramarao, von Andrian et al. 1994; Bochner 1997; Matsumoto, Sterbinsky et al. 1997). After stimulation with IL-1, TNF and IL-4, epithelial cells acquire adhesive properties, involving E-selectin (Kyan-Aung, Haskard et al. 1991), P-selectin (Wein, Sterbinsky et al. 1995), intracellular adhesion molecule 1 (ICAM1) (Bochner, Luscinskas et al. 1991) and vascular cell adhesion molecule 1 (VCAM1) (Dobrina, Menegazzi et al. 1991). Interaction of eosinophils with these vascular adhesion molecules requires eosinophil cell surface receptors such as L-selectin (Knol, Tackey et al. 1994), PSGL-1 (Symon, Walsh et al. 1994) and  $\alpha4$  (Walsh, Mermoud et al. 1991) and  $\beta2$  integrins (Nakajima, Sano et al. 1994) e.g the  $\alpha4\beta1$  integrin also known as very late antigen 4 (VLA4) (figure 1.2).

Innate like natural killer T cells (NKT) appear to amplify allergic responses in the presence of  $T_H2$  cells and also drive AHR in the absence of adaptive immune responses (Kim, DeKruyff et al. 2010; Akbari, Stock et al. 2003). NKT cells constitute a subset of lymphocytes with qualities of both T cells and NK cells. The iNKT cells express an invariant T cell receptor (TCR), which behaves like a pattern recognition receptor; recognising glycolipid antigens, instead of conventional peptide antigens. Stimulation of TCR on NKT cells leads to rapid production of such inflammatory cytokines as IL-4, IL-13 and IFN- $\gamma$  required later for the adaptive



response (Lloyd and Hessel 2011; Taniguchi, Harada et al. 2003; Taniguchi, Seino et al. 2003).

The natural helper cell (also named nuocyte or multipotent progenitor cell), has been identified in fat-associated lymphoid clusters (Moro, Yamada et al.) and mesenteric lymph nodes from helminth infected mice (Neill, Wong et al. ; Saenz, Siracusa et al.). Natural helper cells respond to IL-25 and IL-33 and produce  $T_H2$  cytokines in the innate response (Moro, Yamada et al. 2011; Neill, Wong et al. 2011; Saenz, Siracusa et al. 2011). These multipotent progenitor cells show expression of c-Kit, IL-7R, IL-33R and IL-17RB and MHC class II and costimulatory molecules; with the advantage of differentiating into monocyte, macrophage, mast, eosinophils or basophils in the presence of stem cell factor (SCF) and IL-3 (Saenz, Siracusa et al. 2011). Given that both IL-25 and IL-33 induce AHR without added stimulation, it is possible that natural helper cells contribute to this response in the absence of  $T_H2$  cells (Kim, DeKruyff et al. 2011).



**Figure 1.2 The Adaptive response in airway inflammatory disease.** Initial sensitisation involves activation and maturation of antigen presenting dendritic cells in response to thymic stromal lymphopoietin (TSLP), produced by epithelial, fibroblasts and mast cells. Mature dendritic cells induce differentiation of naïve CD4<sup>+</sup> T cells to T<sub>H</sub>2 cells which produce IL-4, IL-5, IL-9, IL-13 and tumour necrosis factor (TNF). IL-4 promotes the differentiation and proliferation of T cells and switching of B cells from IgG to IgE production to target mast cells for degranulation. IL-5 plays a role in eosinophil differentiation, maturation and survival. IL-13 plays a role in AHR and mucus production. IL-17 mediates neutrophil infiltration by inducing bronchial epithelial cells to produce CXC chemokines and G-CSF (a survival and proliferation factor). Epithelial cells also release stem cell factor (SCF), required for maintaining mucosal mast cells at the airway surface and CCL11 which recruits eosinophils via CCR3. Tregs negatively regulate the T<sub>H</sub>2 response through IL-10 and transforming growth factor- $\beta$  (TGF- $\beta$ ). Adapted from (Levine and Wenzel 2011; Barnes 2008; Holgate and Polosa 2008). MHC class II: major histocompatibility complex class II, VLA4: very late antigen 4, VCAM1: vascular cell adhesion molecule 1, TCR: T-cell receptor.

### 1.1.2 The adaptive response

In asthma, the induction of the adaptive immune response involves uptake of allergens by antigen presenting cells (APC), such as DC (Iwasaki and Medzhitov

2004) located immediately above the basement membrane of the airway epithelium which have direct contact with invading allergens (Jahnsen, Moloney et al. 2001). DC originate from CD34+ bone marrow progenitor cells or CD14+ monocytes and differentiate into immature DC (Finn and Bigby 2009). DC maturation is stimulated by necrotic cells, cytokines, activated T cells that express CD40 ligand and innate immune response ligands derived from microbes. These components lead to reduced endocytosis and up regulation of T cell co-stimulatory molecule expression including CD80, CD86 and CD40 on APCs in addition to MCH class II expression and chemokines (CCL17, CCL22) which attract T cells, eosinophils and basophils to the lungs (Lambrecht and Hammad 2009). During allergic airway inflammation DC levels are increased in the lung and regional lymph nodes (Lambrecht and Hammad 2003) and peripheral blood (Matsuda, Suda et al. 2002). CD40 and CD86 expression is also increased on DC at the site of inflammation (Vermaelen and Pauwels 2003). Antigen bearing mature plasmacytoid DCs migrate away from the airway wall to regional lymph nodes (Huh, Strickland et al. 2003) then stimulate naïve T cells to produce a T<sub>H</sub>2 profile (Rissoan, Soumelis et al. 1999).

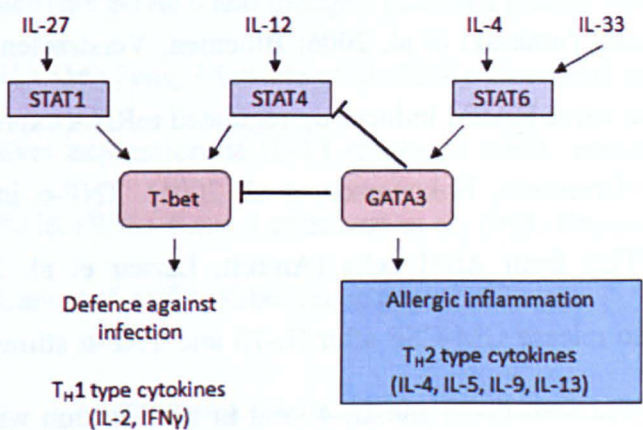
T<sub>H</sub>2 cells then coordinate upregulated expression of a cluster of genes on human chromosome 5q31-33 which include genes encoding IL-4, IL-5, IL-9, IL-13 and GM-CSF (Cousins, Lee et al. 2002; Barnes 2008). IL-4 acts through STAT6 to regulate GATA3 (GATA 3 binding protein) (Barnes 2008). An increased population of GATA3 T cells is noted in asthmatic airways compared to normal airways (Nakamura, Ghaffar et al. 1999). Following phosphorylation and activation by mitogen activated protein kinase (MAPK) p38, GATA3 translocates from the cytoplasm to the nucleus to activate gene transcription (Maneechotesuwan, Xin et al. 2007), which mediates cytokine secretion (IL-4, IL-5, IL-13, IL-9) and also inhibits

the actions of T-bet (which acts to induce IL-12) via inhibition of STAT4 (Usui, Preiss et al. 2006). IL-9 attracts and drives mast cell differentiation (Kay 2006), IL-5 drives eosinophil differentiation in the bone marrow and further maturation and survival (O'Byrne 2007). IL-4 promotes the differentiation and proliferation of T<sub>H</sub>2 cells and drives IgE production by B cells (Avila 2007). Cross linking of FcεRI by IgE bound antigen triggers mast cell degranulation. IL-13 plays a role in airway hyper-reactivity and mucus hyper-production (Wills-Karp and Finkelman 2008). Polymorphisms in IL-4, the IL-4 receptor α chain (IL-4Rα) and IL-13 are consistently observed in human asthma (Ober and Hoffjan 2006). Polymorphisms in both IL-4 and IL-4Rα have been associated with severe asthma (Sandford, Chagani et al. 2000; Wenzel, Balzar et al. 2007) and with increased airway mast and IgE cells (Balzar, Strand et al. 2007). IL-3, IL-4, IL-5 and GM-CSF are involved with maturation of eosinophils and basophils (Hammad and Lambrecht 2008) (see figure 1.2). Stimulated by the T<sub>H</sub>17 cells, IL-17 is also suggested to play a role in neutrophilic inflammation and steroid resistance in a murine model of asthma (McKinley, Alcorn et al. 2008). The recently discovered IL-33 appears to promote T<sub>H</sub>2 cell differentiation by translocating to the nucleus and regulating transcription through an effect on chromatin structure (Carriere, Roussel et al. 2007). In addition to TSLP, IL-25 and IL-33, the epithelium produces CCL11, CCL17, CCL22. These chemokines act on the chemokine receptor CCR3 on eosinophils and CCR4 on T<sub>H</sub>2 cells, to amplify the T<sub>H</sub>2 response (Holgate 2007; Schleimer, Kato et al. 2007).

Treg (T regulation) cells provide inhibition by producing anti-inflammatory cytokines such as IL-10 and TGF-β (Ostroukhova, Seguin-Devaux et al. 2004; Kearley, Barker et al. 2005) and by expressing inhibitory molecules such as cytotoxic T lymphocyte antigen4 (CTLA4) and also induce down regulation of MHC

class II and the costimulatory molecules CD80 and CD86 by APC (Cederbom, Hall et al. 2000). Rather than produce IL-10 directly, Treg can induce nearby CD4+ T cells to express IL-10 (Kearley, Barker et al. 2005). For suppression of DC, Treg cells form lymphocyte function associated antigen 1 (LFA1) dependent aggregates with DC which results in down regulated DC costimulatory molecules in response to CTLA4 (Onishi, Fehervari et al. 2008).

$T_H1$  cell differentiation also plays a role in defence against infection. Through STAT1 and STAT4, T-bet induces production of IL-2 and interferon- $\gamma$  (IFN- $\gamma$ ) (Barnes 2008). Notably T-bet is reduced in airways from asthmatic patients compared with non-asthmatic subjects (Finotto, Neurath et al. 2002). T-bet serves as an important regulator of GATA3. When phosphorylated T-bet can associate and inhibit GATA3 function by preventing it from binding to its target DNA sequences (Hwang, Szabo et al. 2005) (figure 1.3).



*Figure 1.3 The  $T_H1/ T_H2$  balance in asthma (described in text, adapted from (Barnes 2008)).*



### 1.1.3 ASM cells and asthma pathogenesis

ASM is a therapeutic target in asthma given its role in regulating airflow obstruction, however, accumulating research suggests ASM may contribute to the pathophysiology of asthma in the airways through changes in airway wall structure, abnormal resistance to relaxation and maintenance of airway inflammation. The prevalence of inflammation in bronchoscopic biopsies and post mortem studies of asthmatics showing bronchial eosinophilic infiltrates has prompted research into the mediators involved in inflammation and the structural implications on contraction from airway remodelling.

Evidence for the immunomodulatory effects of ASM comes from studies showing that *in vivo* human ASM (HASM) cells can release IL-1 $\beta$ , IL-5, IL-6, GM-CSF, CCL11 and RANTES (regulated upon activation normal T cells, which is a CC chemokine with chemo-attractant activity for eosinophils, memory T cells and monocytes) (Ammit, Lazaar et al. 2002; Grunstein, Hakonarson et al. 2002; Hirst, Hallsworth et al. 2002; Moore, Church et al. 2002; Halayko and Amrani 2003; Tran, Fernandes et al. 2005; Rahman, Yamasaki et al. 2006; Bloemen, Verstraelen et al. 2007). IL-10 administration on naïve HASM induces up regulated mRNA expression and release of IL-5 protein (Grunstein, Hakonarson et al. 2001). TNF- $\alpha$  induces secretion of IL-6 and RANTES from ASM cells (Ammit, Lazaar et al. 2002). Cultured airway myocytes also release GM-CSF after IL-1 $\beta$  and TNF- $\alpha$  stimulation (Saunders, Mitchell et al. 1997). Both IL-13 and IL-4 (and in combination with IL-1 $\beta$ ) induce CCL11 (eotaxin) release by ASM, mediated by IL-4R- $\alpha$  (Hirst, Hallsworth et al. 2002). Both IL-9 and IL-17A mediated CCL11 release from ASM requires the STAT3 pathway (Yamasaki, Saleh et al. ; Saleh, Shan et al. 2009). IL-5

also acts on ASM to induce IL-1 $\beta$  and other IL-1 members (Hakonarson, Maskeri et al. 1999; Hakonarson, Whelan et al. 2002; Whelan, Kim et al. 2004). High affinity (Fc $\epsilon$ RI) and low affinity (Fc $\epsilon$ RII, also known as CD23) receptors are expressed on HASM (Delespesse, Sarfati et al. 1992; Sarfati, Fournier et al. 1992; Bonnefoy, Lecoanet-Henchoz et al. 1997; Kinet 1999). Following IgE sensitisation, HASM cells release CCL11 and IL-5. This release is inhibited by an anti-Fc $\epsilon$ RI  $\alpha$ -chain antibody (Ab) targeting the IgE binding site (Hakonarson, Carter et al. 1999; Grunstein, Hakonarson et al. 2002; Gounni 2006). Fc $\epsilon$ RI activated transient increases in intracellular Ca<sup>2+</sup> in HASM cells have also been reported (Gounni, Wellemans et al. 2005). The increased contractility/ attenuated relaxation seen after activation of Fc $\epsilon$ RII by IgE complexes and atopic asthmatic serum is also inhibited by an anti-CD23 neutralising Ab (Hakonarson and Grunstein 1998).

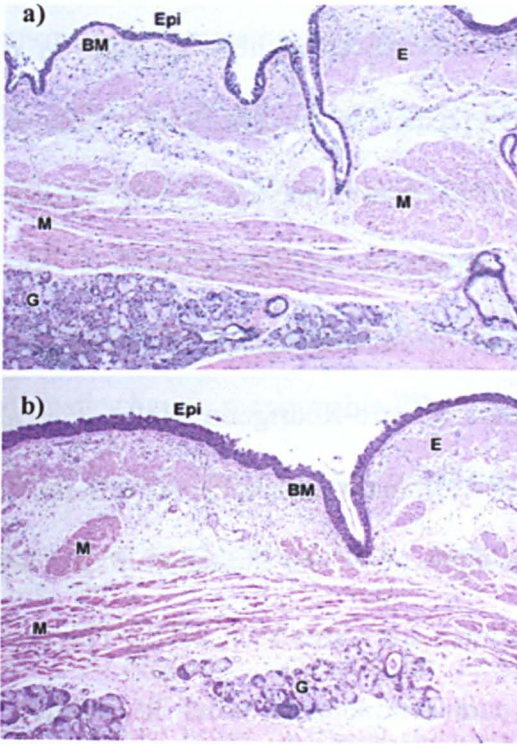
Inflammatory mediators may influence the contractile response in HASM. In cultured cells both IL-13 and IL-4 act through the IL-4 type II receptor, a dimer that consists of IL-4R $\alpha$  and IL-13R $\alpha$  (Laporte, Moore et al. 2001). Both IL-4 and IL-13 activate STAT6 and mitogen activated protein kinase (MAPK) signalling cascades in HASM (Peng, Matsuda et al. 2004). In animal models exogenous administration or over expression of IL-13 results in AHR, while inhibition of IL-13 decreases this AHR (Wills-Karp, Luyimbazi et al. 1998; Kuperman, Huang et al. 2002; Venkayya, Lam et al. 2002; Kibe, Inoue et al. 2003).

ASM plays a role in the deposition of extracellular matrix proteins (Johnson et al., 2000), by releasing fibronectin and collagen. ASM also promotes inflammatory cell recruitment through expression of cell surface adhesion molecules (Black et al., 2001). Vascular cell adhesion molecule 1 (VCAM1) and intracellular adhesion

molecule 1 (ICAM1) are expressed on cultured HASM and further induced after exposure to IL-1 $\beta$ , TNF- $\alpha$  and IL-13 (Panettieri, Lazaar et al. 1995; Syed, Panettieri et al. 2005; Wang, Lin et al. 2005). This can potentially facilitate binding of T cells (Lazaar, Albelda et al. 1994) and eosinophils (Hughes, Arthur et al. 2000) to airway myocytes (Hershenson, Brown et al. 2008). IL-1 $\beta$  and TNF- $\alpha$  stimulation of cultured HASM increases activity of cyclooxygenase-2 (COX-2) (Belvisi, Saunders et al. 1997) which can lead to increased levels of proinflammatory prostanoids. Interferon  $\beta$  attenuates TNF- $\alpha$  induced RANTES expression by HASM (Tliba, Tliba et al. 2003). The growth factor TGF- $\beta$  stimulates HASM hypertrophy (McKay, de Jongste et al. 1998). IL-4, IL-5, IL-13 induce expression of vascular endothelial growth factor (VEGF) in HASM, HASM also express VEGF receptors (Wen, Liu et al. 2003; Kazi, Lotfi et al. 2004). VEGF induces fibronectin expression, which has been suggested to promote ASM mitogenesis.

In asthma changes due to inflammation and remodelling can be seen throughout the airway generations (Jeffrey 1998; Bousquet, Jeffery et al. 2000). An increase in ASM layer thickness is a consistent feature in histological sections from asthmatic airways (Black and Johnson 2002) particularly patients with fatal asthma (Ebina, Yaegashi et al. 1990; Kuwano, Bosken et al. 1993). Studies of force generation in asthmatic airways versus healthy controls produce conflicting results. However, the rare studies assessing isotonic shortening in general show increased shortening in asthmatic airways compared to controls (Bramley, Thomson et al. 1994; Thomson, Bramley et al. 1996).





**Figure 1.4** Histological staining in central airway walls from a) asthmatic and b) normal subjects. The asthmatic airway shows epithelial disruption (Epi) and thickening of the subepithelial basement membrane (BM). Airway smooth muscle mass is increased (M) and mucus gland (G) hypertrophy is shown. Original magnification is 100X. Images provided by Dr. Aliya N. Husain. Images taken from (Hershenson, Brown et al. 2008).

#### 1.1.4 Diagnosis and treatment of asthma

The developing respiratory system is susceptible to inflammatory damage during early life; studies suggest the risk of future asthma development relates to initial age of allergic sensitisation (Peat, Toelle et al. 1996; Sherrill, Stein et al. 1999). Although most wheezing in the immediate postnatal period is due to small airway diameter (Stein, Sherrill et al. 1999), studies indicate that early infections (Stein, Sherrill et al. 1999) and sensitisation to inhaled allergens (Custovic, Simpson et al. 2001) in later infancy and early childhood (during first three years of life) (Patino and Martinez 2001; Moller, Dreborg et al. 2002) are associated with an increased risk of asthmatic symptoms and potential future asthma development (Holt and Sly 2002). In addition, programming of T cell memory against environmental allergens (such as triggers for asthma) occurs early in life (Holt and Sly 2002). A predictive index has been produced for development of persistent asthma in children

less than three years of age who have experienced multiple episodes of wheezing during the previous 12 months. Positive predictions correlate with at least one of the following risk factors: major criteria i) parental history of asthma, sensitisation to aeroallergens, diagnosis of atopic dermatitis or, minor criteria ii) allergic sensitisation to food,  $\geq 4\%$  peripheral blood eosinophilia, diagnosis of allergic rhinitis or wheezing unrelated to colds (Castro-Rodriguez, Holberg et al. 2000; Guilbert, Morgan et al. 2006). Childhood asthma consists of many different subtypes however atopy associated asthma typically persists into adulthood (Peat, Toelle et al. 1996; Ernst, Ghezzo et al. 2002; Kjellman and Gustafsson 2002). Therefore initial treatment is designed to provide acute symptomatic relief in children. Future therapeutic approaches involve minimising long term inflammatory damage to the growing lungs that may result from persistent asthma into adulthood (Holt, Sly et al. 2004).

For a clinical diagnosis of asthma, a detailed medical history and full medical examination is required including pulmonary function testing (for patients over five years). A spirometry test is used to demonstrate the degree of airway obstruction and to determine whether it is reversible by measuring the forced expiratory volume in one second ( $FEV_1$ ) expressed as a percentage of the forced vital capacity (FVC, the maximal volume of air exhaled after maximal inhalation) (or if held over six seconds,  $FEV_6$ ) (Bye, Kerstein et al. 1992; Li and O'Connell 1996; Llewellyn, Sawyer et al. 2002). This may include testing before and after the patient inhales a short acting bronchodilator. Results are compared to a predictive baseline measurement based on age, weight and height of the patient. An annual reduction in  $FEV_1$  is associated with increased episodes of wheezing or shortness of breath (Fuhlbrigge, Kitch et al. 2001; Fuhlbrigge, Weiss et al. 2006). An additional

methacholine) for d

2007) (see example

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Levine and Wenzel ;



Corticosteroids are the most effective therapy for chronic persistent asthma (Barnes 2006). They suppress  $T_H2$  mediated inflammation through the inhibition of expression of cytokines, chemokines and adhesion molecules (Barnes and Adcock 2003). Free corticoids diffuse across the cell membrane and bind to cytoplasmic glucocorticoid receptors. This causes translocation to the nucleus and results in altered transcription of target genes. In addition through the transactivation pathway the glucocorticoid receptor binds to the glucocorticoid response element (GRE) in the promoter region of steroid sensitive genes leading to transcription of genes for anti-inflammatory mediators (inhibitor of nuclear factor- $\kappa$ B ( $I_{\kappa}B\alpha$ )). During transrepression the glucocorticoid receptor and GRE complex with histone acetyltransferase (HAT) activity switch off expression of the inflammatory genes. Although asthma is generally classed as a  $T_H2$  response; recent gene expression studies in mild asthma suggest that the  $T_H2$  profile is only shown in 50% of patients with asthma. This group was characterised by more AHR, eosinophils and remodelling. The  $T_H2$  profiled patients responded well to inhaled corticosteroids, where as the non- $T_H2$  signature patients did not (Woodruff, Modrek et al. 2009).

Inhaled short acting  $\beta_2$ -adrenoreceptor agonists (SABA) such as salbutamol and terbutaline are used as bronchodilators for rapid relief. The long acting  $\beta_2$ -adrenoreceptor agonists (LABA) formoterol and salmeterol provide relief for up to 12 hours. The  $\beta_2$ -adrenoreceptor is in an active form when it associates with the  $\alpha$  subunit of the  $G_s$  protein together with a molecule of guanine triphosphate (GTP). The replacement of GTP with guanine diphosphate (GDP) catalyses the conversion of ATP to cAMP and reduces the affinity of the  $\alpha$  subunit for the receptor, to return to its disassociated, inactive form. cAMP catalyses the activation of protein kinase A (PKA). cAMP also inhibits release of  $Ca^{2+}$  from internal stores, reduces  $Ca^{2+}$  entry

into cells and induces sequestration of intracellular  $\text{Ca}^{2+}$ , which leads to airway relaxation (discussed in detail in section 1.2).  $\beta_2$ -adrenoreceptor agonists may act to temporarily stabilise the receptors in their active state (bound to  $\text{G}_s\text{-GTP}$ ) (Onaran, Costa et al. 1993). Combination inhalers for corticosteroids and LABAs have been introduced for convenience to increase patient adherence (Palmqvist, Persson et al. 1997; Usmani, Ito et al. 2005). There is also evidence of uncoupling of  $\beta_2$ -adrenergic receptors in the airways of patients who die from asthma (Bai, Mak et al. 1992).

Allergen specific immunotherapy has been trialled for the treatment of mild asthma, allergic rhinitis and selected allergies. Immunity tolerance is induced through repeated allergen exposure, leading to an increase in allergen specific IgA and IgG4 antibodies (Ab) and a decrease in the level of IgE Ab. Immunotherapy requires regular subcutaneous injection of allergen extracts or recombinant allergens, involving a build up phase (usually weekly injections) and a maintenance phase (monthly injections). However anaphylactic side effects may occur and the efficiency of this approach remains uncertain (Williams, Krishna et al. 2004).

Mast cell inhibitors such as sodium cromoglicate were first introduced as an asthma therapy in 1968 (Edwards and Howell 2000), followed by nedocromil sodium. After inhalation the drugs inhibit early and late phase responses in the upper and lower airways (Bradding, Walls et al. 2006). However, many mast cell stabilising drugs are ineffective at inhibiting mast cell activation in chronic asthma.

Anti-IgE therapies such as the humanised Ab Omalizumab have been shown to reduce serum levels of free IgE and down regulate expression of IgE receptors ( $\text{Fc}\epsilon\text{RI}$ ) on mast cells and basophils. Tissue eosinophilia, B and T cells are reduced also. Omalizumab decreases  $\text{Fc}\epsilon\text{RI}$  expression on circulating dendritic cells, possibly

reducing further  $T_H2$  cell activation (Holgate, Casale et al. 2005). IgE Ab specific for the C3 domain of IgE that block IgE binding to FcεRI, FcεRII and CD23 lead to inhibition of the inflammatory response in human and mouse airways (Corne, Djukanovic et al. 1997). Clinical trials with patients with poorly controlled IgE mediated asthma receiving subcutaneously administered Omalizumab showed improved symptom control and allowed patients to receive lower doses of inhaled corticosteroids (Holgate, Djukanovic et al. 2005). The efficacy of Omalizumab on asthma exacerbations was unaffected by patient age, gender, baseline serum IgE or by the 2/4 weekly dosing schedule (Bousquet, Cabrera et al. 2005). Lumiliximab, a low affinity anti- IgE Fc receptor also went through to phase II studies (Ballow 2006).

Anti-cytokine targeting therapies investigated include altrakincept, a soluble recombinant human IL-4R. Initial trials showed promise as inhaled nebulized altrakincept over 12 weeks allowed patients to withdraw treatment with corticosteroids without relapse (Borish, Nelson et al. 1999). However, phase three trials failed to confirm efficacy for this treatment in asthma. Pitrankinra, is an IL-4 variant which inhibits the binding of IL-4 and IL-13 to the IL-4R  $\alpha$  subunit; pitrankinra reduced allergen induced late phase response in asthmatic patients (Wenzel, Wilbraham et al. 2007). The humanised anti-IL-4 Ab, Pascolizumab (Hart, Blackburn et al. 2002) has also been studied. However, in a study using grass pollen, it was reported that the secondary IgE response is not T cell dependent, raising doubts on the use of IL-4 inhibition in established disease (Linhart, Bigenzahn et al. 2007). Phase III studies of the anti-IL-5 agent Mepolizumab have shown a reduction in exacerbations and required oral corticosteroid dose in refractory eosinophilic asthma (Halдар, Brightling et al. 2009; Nair, Pizzichini et al.

2009). Currently MEDI-528, a humanised, monoclonal anti-IL-9 Ab is being investigated in early phase trials for asthma (Antoniou 2011). IMA-638, a humanized IL-13 monoclonal Ab inhibited both early and late allergen challenge response (Gauvreau, Boulet et al. 2010). Clinical trials have now been completed, evaluating the safety of CAT-354 (a humanized IgG<sub>1</sub> anti-IL-13 Ab) (Singh, Kane et al. 2010) and AMG 317 (a human monoclonal IL-4R $\alpha$  Ab which inhibits both IL-4 and IL-13 pathways) (Corren, Busse et al. 2010). The humanised mouse anti-TNF- $\alpha$  Infliximab, showed benefit in conjunction with inhaled corticosteroids for asthma treatment in phase II trials (Cazzola and Polosa 2006; Erin, Leaker et al. 2006). Etanercept, an IgG<sub>1</sub>-TNF p75 receptor fusion protein showed a small but significant improvement in asthma control and systemic inflammation (Morjaria, Chauhan et al. 2008). Golimumab, the human monoclonal anti-TNF- $\alpha$  Ab produced serious adverse effects (Wenzel, Barnes et al. 2009) (refer to table 1.1 for summary). In addition, TNF- $\alpha$  may contribute to muscarinic M2 receptor dysfunction and airway hyper-reactivity by decreasing receptor expression and promoting recruitment of eosinophils, containing major basic protein, an M2 antagonist. In a study showing antigen challenged animals with hyper-reactivity to vagus stimulation and dysfunctional neuronal M2 receptors; Entanercept prevented M2 receptor dysfunction and airway hyper-reactivity and reduced eosinophil infiltration (Nie, Jacoby et al. 2009). An anti-CD4, anti-CD11a and anti-IL-2  $\alpha$  chain are currently in clinical trials (Ballow 2006). Based on its role in recruitment of eosinophils and mast cells, CCL11/CCR3 has been considered for therapeutic targeting (Dent, Hadjicharalambous et al. 2004; Garcia, Godot et al. 2005).

Despite the positive findings or specific end points in some of the above studies, the general utility of anti-cytokine approaches in the treatment of asthma remains to be determined.

**Table 1.1 Summary of anti-cytokine therapy clinical trials for asthma. Ab: antibody.**

Target	Therapeutic intervention	Reference
<b>IL-4</b>	Altrakincept, recombinant human IL-4R as IL-4 antagonist (inhaled)	(Borish, Nelson et al. 2001)
	Pitrakinra, IL-4 variant, inhibits binding of IL-4 and IL-13 to IL-4R $\alpha$ receptor complexes (intravenous and inhaled)	(Antoniou ; Wenzel, Wilbraham et al. 2007)
	Pascolizumab (SB240683), humanised anti-IL-4 Ab	(Hart, Blackburn et al. 2002)
	AMG 317, human monoclonal IL-4R $\alpha$ Ab, inhibits both IL-4 and IL-13 (intravenous)	(Corren, Busse et al. 2010)
<b>IL-5</b>	mepolizumab, humanised monoclonal anti-IL-5 Ab (intravenous)	(Flood-Page, Swenson et al. 2007; Haldar, Brightling et al. 2009; Nair, Pizzichini et al. 2009)
	SB-240563, humanised monoclonal IL-5 Ab (intravenous)	(Leckie, ten Brinke et al. 2000)
	SCH55700, humanised anti-human IL-5 Ab (intravenous)	(Kips, O'Connor et al. 2003)
<b>IL-9</b>	MEDI-528, humanised, monoclonal anti-IL-9 Ab (intravenous)	(Antoniou ; Desai and Brightling 2009)
<b>IL-13</b>	CAT-354, human IgG <sub>4</sub> anti-IL-13 Ab (intravenous)	(Singh, Kane et al. 2010)
	IMA-638 and IMA-026, humanised IgG <sub>1</sub> anti IL-13 Ab (intravenous)	(Gauvreau, Boulet et al. 2010)
<b>TNF-<math>\alpha</math></b>	Etanercept, a soluble fusion protein combining two p75 TNF receptors with an Fc fragment of human IgG1 (intravenous)	(Howarth, Babu et al. 2005; Berry, Hargadon et al. 2006; Morjaria, Chauhan et al. 2008)
	Infliximab, a chimeric mouse/humanised monoclonal anti-TNF- $\alpha$ Ab (intravenous)	(Erin, Leaker et al. 2006)
	Golimumab, human monoclonal anti-TNF- $\alpha$ Ab (intravenous)	(Wenzel, Barnes et al. 2009)



Gene silencing through antisense oligonucleotides and small interfering RNAs (siRNAs) has been considered for post-transcriptional mRNA inhibition. siRNA against NF- $\kappa$ B by transfection of airway epithelial cells reduced TNF- $\alpha$ -induced IL-6 and IL-8 release (Platz, Pinkenburg et al. 2005). NF- $\kappa$ B is a transcription factor with regulatory roles for the expression of many cytokines, chemokines and adhesion molecules (Baldwin 2001). In resting cells NF- $\kappa$ B is inactive in the cytoplasm, bound to an inhibitory I- $\kappa$ B protein. With stimulation, the I- $\kappa$ B protein is phosphorylated by an I- $\kappa$ B kinase complex consisting of the catalytic subunits IKK-1, IKK-2 and a regulatory IKK- $\gamma$  subunit (Scheidereit 1998; Karin 1999; Courtois, Smahi et al. 2001). With I- $\kappa$ B removed, the transcription factor translocates to the nucleus to activate gene transcription. Using the NF- $\kappa$ B inhibitor IKK-2 resulted in a reduction in the inflammatory response in primary human ASM cells and an antigen driven rat model of lung inflammation (Birrell, Hardaker et al. 2005). Other inflammatory targets include MAPK (JNK, ERK, p38) as downstream pathways influence cytokine and chemokine expression (Schieven 2005) and cell survival (Manke, Nguyen et al. 2005). SB-6B1323, a p38 inhibitor made phase II trials for COPD, rheumatoid arthritis and coronary disease (Behr, Berova et al. 2003).

Impaired barrier function as a result of defective tight junction assembly has been considered for trials to restore barrier function through growth factor administration. Keratinocyte growth factor has been shown to induce epithelial cell proliferation and significantly limited the allergen induced alterations in epithelium integrity in ovalbumin sensitised rats (Tillie-Leblond, Gosset et al. 2007).

Another approach is Bronchial Thermoplasty (Castro, Musani et al. 2010; Castro, Rubin et al. 2010). This involves delivering radiofrequency generated heat to the airways via a catheter inserted in the bronchial tree using a flexible bronchoscope; to reduce smooth muscle quantity and contractility (Cox, Miller et al. 2004). The results showed improved peak expiratory flow, symptom-free days and improved AHR (Cox, Miller et al. 2006). However, the long term side effects are not yet known and the airway burning can lead to mucous production and asthma exacerbations.

## 1.2 MECHANISMS OF AIRWAY SMOOTH MUSCLE CONTRACTION

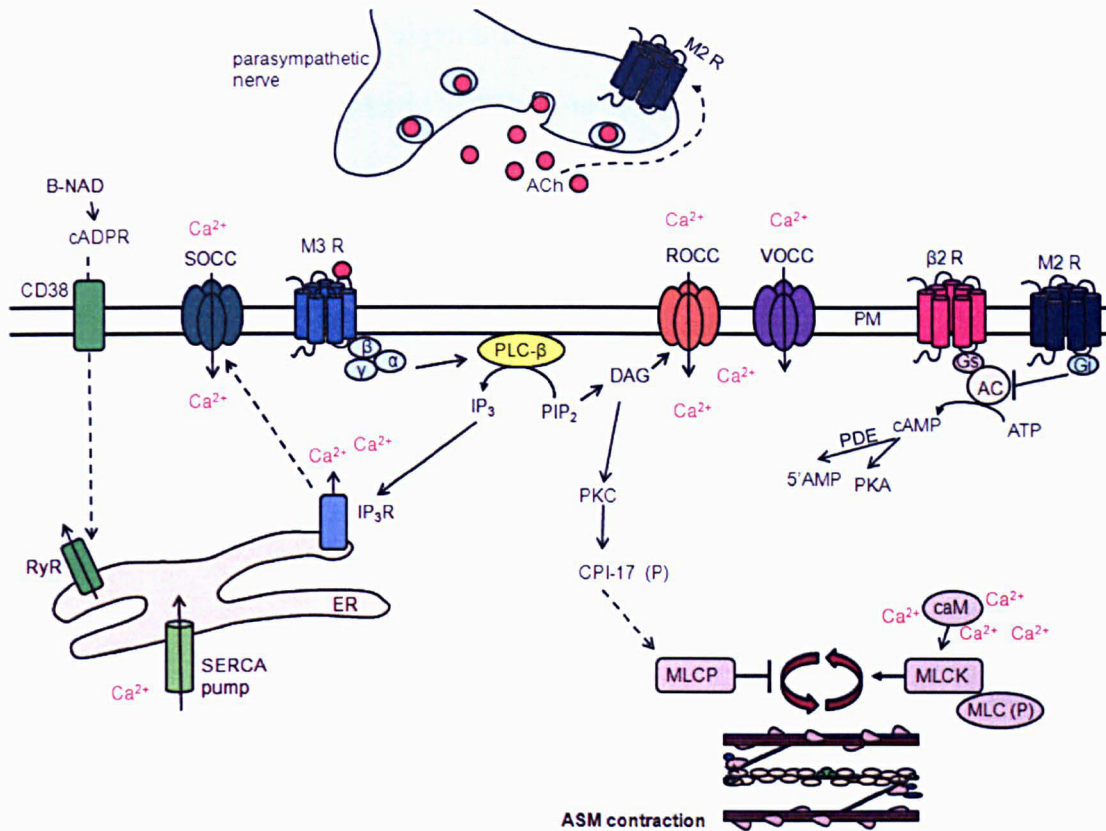
### 1.2.1 $\text{Ca}^{2+}$ signalling pathways for ASM contraction

As discussed above, asthma is characterised by an increase in airway resistance, which is contributed to by abnormal contraction of the ASM (Barnes 1998). ASM tone is primarily controlled by the release of neuronal acetylcholine (ACh) on the parasympathetic nerves via the muscarinic receptors. Of the five muscarinic receptor subtypes (M1-5), ASM cells express M2 and M3 (Mak and Barnes 1990), the less abundant M3 receptors are responsible for tracheal and bronchial contraction (Roffel, Elzinga et al. 1990) via the PLC pathway described below (Roffel, Elzinga et al. 1990). Release of ACh is controlled by inhibitory M<sub>2</sub> muscarinic receptors (Fryer and Jacoby 1998). The M<sub>2</sub> receptor has been shown to be dysfunctional in models of airway disease (ten Berge, Santing et al. 1995; Coulson and Fryer 2003). In addition, the M<sub>2</sub> receptors on ASM are coupled via an inhibitory G protein (G<sub>i</sub>) for inhibition of adenylyl cyclase (AC) and might counteract the bronchodilator effects of such drugs as  $\beta_2$ -adrenergic receptor agonists, that require activation of adenylyl cyclase via G<sub>s</sub> (Sankary, Jones et al. 1988; Fernandes, Fryer et al. 1992; Barnes 1998).

Calcium ( $\text{Ca}^{2+}$ ) serves as a second messenger in a diverse range of cell types, playing roles in gene expression, neurotransmission and contraction. In ASM,  $\text{Ca}^{2+}$  cytoplasmic concentrations are increased through entry across the plasma membrane, or through release from internal  $\text{Ca}^{2+}$  stores in the endoplasmic reticulum (ER) (Berridge, Lipp et al. 2000).  $\text{Ca}^{2+}$  release from stores involves the phosphoinositide (PI) pathway. G-protein linked, or tyrosine kinase plasma membrane receptors activate phospholipase C (PLC), leading to cleavage of phosphatidylinositol 4,5–

bisphosphate ( $\text{PIP}_2$ ) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate ( $\text{IP}_3$ ).  $\text{IP}_3$  serves as a  $\text{Ca}^{2+}$  releasing agent, by activating ER membrane located  $\text{IP}_3$  receptors, this binding results in channel opening and release of  $\text{Ca}^{2+}$  from the ER into the cytoplasm (Berridge 1993).  $\text{Ca}^{2+}$  is also potentially released by cyclic adenosine diphosphate ribose via ryanodine receptors (RyR) (Flynn, Bradley et al. 2001) (see figure 1.6).  $\text{Ca}^{2+}$  is taken up from the cytosol to refill internal stores via the sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) pump.

Activation of the phosphoinositide (PI) pathway also leads to two plasma membrane channel operated mechanisms of  $\text{Ca}^{2+}$  entry. Firstly, the second messengers DAG, arachidonic acid (AA) and  $\text{Ca}^{2+}$  itself (Bird, Aziz et al. 2004), generated downstream of the PI pathway activate receptor operated calcium entry channels (ROCC). In addition, the depletion of ER stores resulting from  $\text{IP}_3$  induced release of  $\text{Ca}^{2+}$  results in activation of plasma membrane  $\text{Ca}^{2+}$  entry through stored operated calcium channels (SOCC) (Putney 1986).

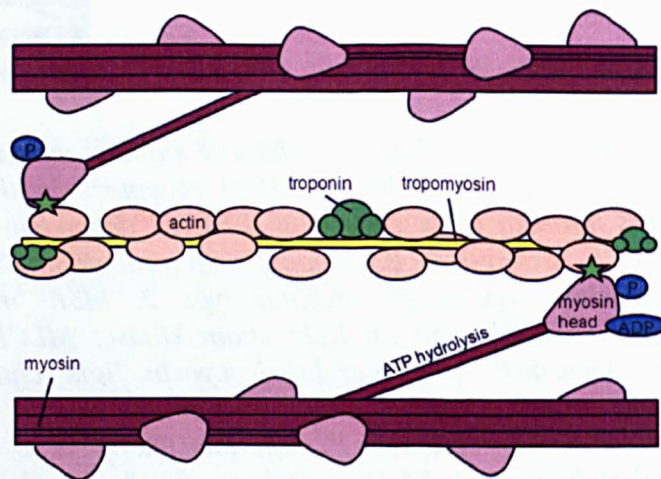


**Figure 1.6.  $\text{Ca}^{2+}$  influx pathways in airway smooth muscle cells, evoked by an agonist acting on the acetylcholine (ACh) receptors in the plasma membrane.** Contraction is initiated by activation of the  $\text{Ca}^{2+}$  dependent phosphoinositide (PI) pathway (refer to text above). ER: endoplasmic reticulum; PM: plasma membrane; M2 R: muscarinic cholinergic receptor type 2; M3R: muscarinic cholinergic receptor type 3; MLCK: myosin light chain kinase; MLCP: myosin light chain phosphatase; myosin-P: phosphorylated myosin light chains; RyR: ryanodine receptor, SERCA: sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase,  $\text{IP}_3$ : inositol triphosphate,  $\text{IP}_3\text{R}$ :  $\text{IP}_3$  receptor, PLC- $\beta$ : phospholipase C, DAG: diacylglycerol,  $\text{PIP}_2$ : phosphatidylinositol 4,5-bisphosphate.  $\beta_2$  R:  $\beta_2$ -adrenergic receptor; AC: Adenyl cyclase; cAMP: cyclic adenosine monophosphate; PDE: phosphodiesterase; PKA: protein kinase A. Adapted from (Dietrich, Chubanov et al. 2006; Pelaia, Renda et al. 2008).

In the cytosol  $\text{Ca}^{2+}$  binds calmodulin which results in the activation of myosin light chain kinase (MLCK). Activated MLCK phosphorylates myosin light chains leading to activation of myosin ATPase, this mediates interactions between myosin and actin; resulting in cross bridging (Kamm and Stull 1986; Word, Tang et al. 1994; Macklem 1996; Gunst and Tang 2000) leading to ASM force development and shortening (figure 1.7). Increased levels of MLC phosphorylation have been



shown in models of inflammation and allergic sensitisation induced AHR (Kong, Halayko et al. 1990; Jiang, Rao et al. 1992; Mitchell, Ndukwu et al. 1993). Myosin light chain phosphatase (MLCP) dephosphorylates myosin light chains and terminates actin/myosin reactions, resulting in smooth muscle relaxation (Barnes 1998). MLCP inhibition also acts on the RyR via the Rho kinase and CPI-17 pathway without impacting on MLCK (Kitazawa, Eto et al. 2000). This allows the degree of contraction to be maintained at a lower  $[Ca^{2+}]_i$ ; a phenomenon known as ‘calcium sensitisation’ (Somlyo and Somlyo 2003) (discussed in 1.2.4).



**Figure 1.7. ASM contraction through myosin-actin cross bridging.** Myosin creates force through mechanical cycles of repeated interaction between the myosin head and actin (Warrick and Spudich 1987). The globular myosin head generates the energy required through enzymatic hydrolysis of adenosine triphosphate (ATP). The binding of ATP to myosin while it is interacting with actin is suggested to lead to conformational changes in the myosin globular head that reduces the affinity for actin and allows it to hydrolyze ATP to adenosine diphosphate (ADP) and inorganic phosphate (P) (Rayment, Smith et al. 1996; Gunst and Tang 2000). The light chain binding domain ('neck region') pivots where the globular head and light chain binding domain touch resulting in separation of the actin from the myosin (Guilford, Dupuis et al. 1997). In smooth muscle the phosphorylation of a serine residue in the N-terminus of the regulatory light chain leads to the cascade for actin activating adenosine triphosphatase (ATPase) activity and contraction (Wagner and Giniger 1981; Sivaramakrishnan and Burke 1982).

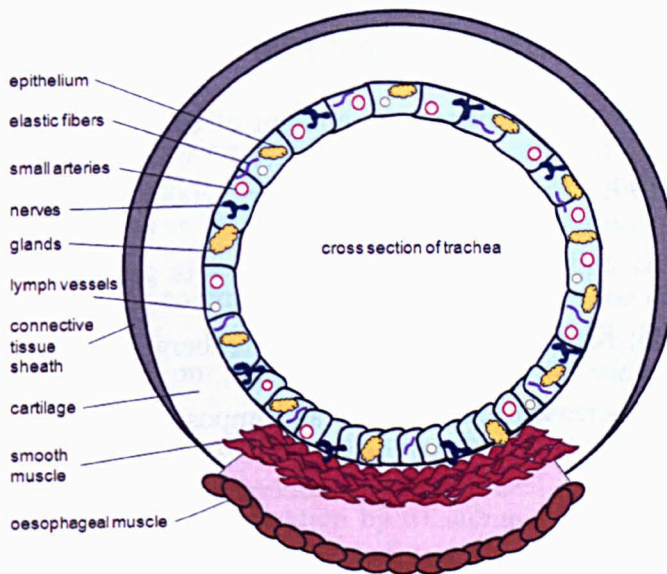
Most ASM relaxation is mediated via  $\beta_2$ -adrenergic receptors (found in all airway generations) (Carstairs, Nimmo et al. 1985) (table 1.2). As mentioned in 1.1.4, the  $\beta_2$ -adrenoreceptor associates with the  $\alpha$  subunit of the  $G_s$  protein together with GTP. The replacement of GTP with GDP catalyses the conversion of ATP to cAMP. After binding, the  $G_s$  dissociates and the  $\alpha$  subunit activates adenylyl cyclase, which breaks down ATP to cAMP. cAMP then catalyses the activation of protein kinase A (PKA) which leads to the dissociation of a catalytic subunit, which then phosphorylates specific cellular targets leading to relaxation (Johnson 1995; Hall 2000; Tanaka, Horinouchi et al. 2005). cAMP also results in inhibition of  $Ca^{2+}$  release from intracellular stores, sequestration of intracellular  $Ca^{2+}$  and reduction of membrane  $Ca^{2+}$  entry contributing to ASM relaxation (Johnson 2006). Although most studies have investigated the cAMP dependent PKA pathway through  $G_s$  coupling,  $\beta_2$  receptors have also been shown to couple to  $G_i$  proteins under some conditions. This can lead to stimulation of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulation kinase (Daaka, Luttrell et al. 1997). This process decreases  $\beta_2$  coupling to  $G_s$ , thus reducing the receptor signal response through this pathway.

**Table 1.2.** Agents known to influence ASM tone, their G-protein coupled receptors and associated G-proteins. \* Not in human airway smooth muscle.

Agonist	G protein coupled receptor	G-protein	Function in ASM	References
<b>Histamine (HA)</b>	H1	G <sub>q</sub>	contraction	(Barnes 2001; Nakayama, Hayashi et al. 2005)
<b>Serotonin *</b> (5-HT)	5-HT <sub>2</sub>	G <sub>q</sub>	contraction	(Barnes 2001; Fanburg and Lee 2000; Tolloczko, Tao et al. 2000)
<b>Endothelin-1,2,3</b>	ET A/B	G <sub>q</sub>	contraction	(Nakayama, Hayashi et al. 2005)
<b>Bradykinin</b>	B <sub>2</sub>	G <sub>q</sub>	contraction	(Adomeit, Graness et al. 1999)
<b>Cysleukotrine</b> <b>LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub></b>	Cys Lt R1	G <sub>q</sub>	contraction	(Barnes 2001)
<b>Acetylcholine (ACh)</b> <b>(neurotransmitter)</b>	M <sub>3</sub>	G <sub>q</sub>	contraction	(Penn and Benovic 2008; Nakayama, Hayashi et al. 2005)
	M <sub>2</sub>	G <sub>i</sub>	inhibits contraction, inhibits adenylyl cyclase	(Penn and Benovic 2008; Unno, Matsuyama et al. 2005)
<b>Noradrenaline</b>	β <sub>2</sub> -adrenergic	G <sub>s</sub> G <sub>i</sub>	relaxation	(Barnes 2001; Kohm and Sanders 2001)

The multilayered airway wall includes the epithelium, basement membrane, mucosa, ASM, cartilage and adventitia with vessels, glands and a network of elastin and collagen throughout layers (Baile, Minshall et al. 1994; Wagner and Mitzner 1996; Wang, Tepper et al. 2000) (figure 1.8). In the trachea hyaline cartilage rings are interrupted by a strip of smooth muscle however, as branching continues down the airway generations the amount of smooth muscle increases while cartilage in the wall decreases and is absent in the distal airways (figure 1.9).

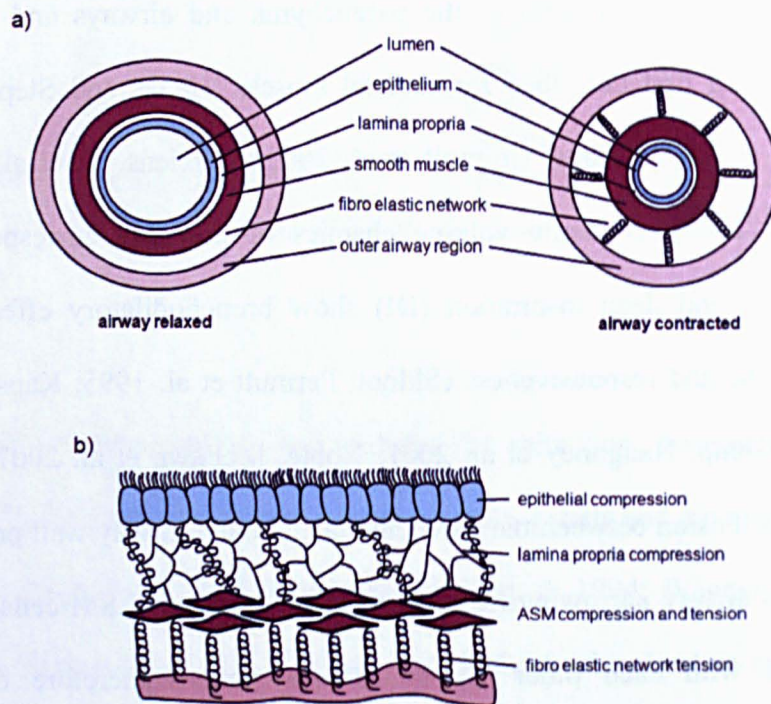




*Figure 1.8. Cross section through trachea showing smooth muscle layer*

During ASM contraction the muscle layer is subjected to compressive stress, while the outer region experiences expansive stress (Mitchell 2009) (figure 1.9). Theories for the poorly understood dynamics of airway contraction have incorporated the stiffness of the parenchyma and airways and the force-velocity relationship first described for skeletal muscle (Hanks and Stephens 1981; Jiang, Rao et al. 1992; Blanc, Coirault et al. 2003; Stephens, Li et al. 2003; Bates and Lauzon 2007). Dynamic volume changes of the lung corresponding with tidal breathing and deep inspiration (DI) show bronchodilatory effects, impacting on ASM tone and responsiveness (Skloot, Permutt et al. 1995; Kapsali, Permutt et al. 2000; Gump, Haughney et al. 2001; Noble, McFawn et al. 2007). When the lung expands tension between the parenchyma and outer airway wall provides a load that opposes airway narrowing (Macklem 1996). Adjacent ASM cells are mechanically coupled with each other via attachment plaques therefore during contractile responses tension is exerted on neighbouring ASM (which impacts on the contraction and shortening of this muscle also) (Skloot, Permutt et al. 1995; King, Moore et al. 1999; Crimi, Pellegrino et al. 2002). These small tidal stretches are large

enough to lead to detachment of the myosin head from actin, sooner than would occur during isometric contraction, reducing the amount of active force generated (Fredberg, Inouye et al. 1999; Fredberg 2000; Fredberg 2000; Fredberg 2001). However for asthmatics the ability of DI to relax airways is greatly diminished (Skloot, Permutt et al. 1995; King, Moore et al. 1999; Fredberg 2001; Scichilone, Pyrgos et al. 2001); due to increased muscle mass and imposed force fluctuations leading to ASM stiffening and less stretch (Fredberg 2000; Fredberg 2000; Mijailovich, Butler et al. 2000; Nguyen and Fredberg 2008). Compared with the effects of thickened submucosal or adventitial layers, a thickened smooth muscle layer is shown to produce the greatest ASM shortening; again supporting the explanation for AHR and the failure of DI to dilate asthmatic airways (Oliver, Fabry et al. 2007).



**Figure 1.9.** During contraction of the distal airways the epithelial, lamina propria and airway smooth muscle (ASM) layers are subjected to compression and tension. The fibro elastic network between the ASM and outer airway wall layers is subjected to tension which contributes to mechanical force for ASM contraction. Adapted from (Cooper, 2009; Mitchell 2009).

### 1.2.2 Plasma Membrane $\text{Ca}^{2+}$ permeable channels

In a wide range of cell types an influx of  $\text{Ca}^{2+}$  is essential for the transduction of functions as varied as action potentials, hormonal release, neurotransmitter release and muscle contraction. In previous decades the classification of  $\text{Ca}^{2+}$  channels has been based on pharmacological response and biochemical properties, however, recent categorisation has incorporated a sequence analysis (Ertel, Campbell et al. 2000) and characterisation by  $\alpha 1$  subunit. Of the  $\text{Ca}^{2+}$  channels classified, most are composed of four-five subunits, the largest of which is the  $\alpha$  subunit which incorporates a conduction pore, voltage sensor and gating apparatus. These are the sites for channel regulation by second messengers, drugs and toxins.

#### 1.2.2.i Voltage Operated Calcium Channels (VOCC)

Of the  $\text{Ca}^{2+}$  permeable channels, VOCC are activated by membrane depolarisation and are highly selective for  $\text{Ca}^{2+}$ , over sodium ions. VOCC regulate such intracellular processes as gene expression, contraction and neurotransmission. In ASM cells the majority of VOCC are L-type in nature. They require a strong depolarization to be activated, are long lasting and are blocked by the L-type  $\text{Ca}^{2+}$  channel antagonists, benzothiazepines, phenylalkylamines and dihydropyridines (Ertel, Campbell et al. 2000). N, P/Q and R-type  $\text{Ca}^{2+}$  currents primarily play a role in neurotransmission and also require strong depolarisation for activation; they are blocked by specific toxins from snail and spider venoms (Neelands, King et al. 2000), rather than the L-type antagonists. T-type  $\text{Ca}^{2+}$  currents are transient and are activated by weak depolarization, they are expressed in many cells types and play a

role in action potentials and firing (Sipido, Carmeliet et al. 1998; Ertel, Campbell et al. 2000). VOCC channels have therapeutic applications for the treatment of abnormal smooth muscle contraction, as in hypertension (Kochegarov 2003). However, inhibitors of VOCC are ineffective bronchodilators in the treatment of asthma (Gordon, Wong et al. 1987), (Patakas, Maniki et al. 1987), (Lofdahl and Barnes 1986). An explanation for the incomplete inhibition of agonist induced contraction is the existence of alternate  $\text{Ca}^{2+}$  entry pathways.

### **1.2.2.ii Receptor Operated Calcium Channels (ROCC)**

The molecular identity of the ROCC is less clear although it is believed to be distinctive from the ER store sensing role of the SOCC (McFadzean and Gibson 2002). Initial studies of ROCC showed that externally applied ATP produced rapid depolarisation of smooth muscle (Suzuki 1985) due to a cation channel with a high selectivity for  $\text{Ca}^{2+}$  (Benham and Tsien 1987). Receptor operated  $\text{Ca}^{2+}$  influx channels are found in excitable and non excitable cells and open rapidly upon binding to an external ligand such as the M2 receptor agonist or the neurotransmitter noradrenaline (Parekh and Putney 2005). ROCC activation involves signalling molecules stimulated through the PLC pathway such as DAG and  $\text{IP}_3$  (Albert and Large 2003; Putney 2007). The G-protein ( $\text{G}_i/\text{G}_o$  sub-group) is suggested to play an important role in this transduction pathway to link the receptor and cation channel in smooth muscle cells (Komori, Kawai et al. 1992; Kim, Kim et al. 1998) and airway myocytes (Wang and Kotlikoff 2000). To further understand the transduction pathway coupling receptors to activation of the ROCC, the distribution and TRP protein combinations for channel formation requires more investigation.

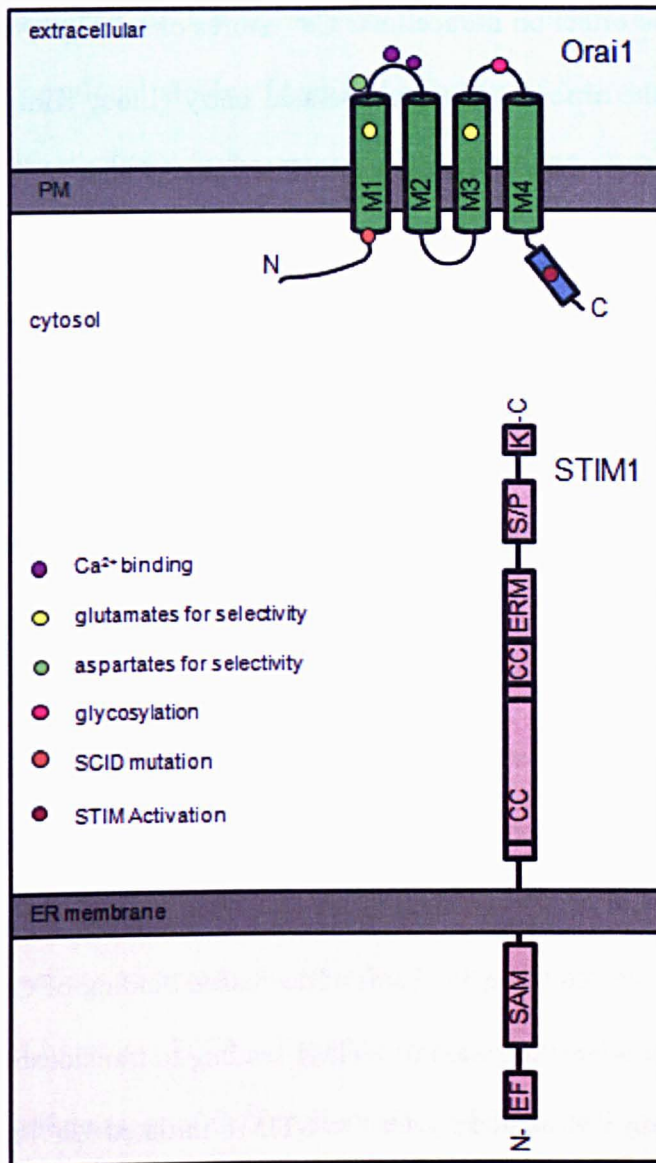


### 1.2.3 Store Operated Calcium Channels (SOCC)

By the mid 1980's it was understood that stimulation of specific plasma membrane receptors lead to  $\text{IP}_3$  activated release of  $\text{Ca}^{2+}$  from non mitochondrial  $\text{Ca}^{2+}$  stores (Berridge and Irvine 1984), in which  $\text{Ca}^{2+}$  release was followed by  $\text{Ca}^{2+}$  entry. This  $\text{Ca}^{2+}$  influx was suggested to be due to a result of depleted ER  $\text{Ca}^{2+}$  stores; introducing the concept of store operated  $\text{Ca}^{2+}$  entry (Putney 1986). This SOCC pathway plays a role in maintaining  $\text{Ca}^{2+}$  levels for the ER and was thought to be important for protein folding and trafficking (Sambrook 1990; Berridge, Lipp et al. 2000).

Over the next 20 years the mechanisms behind the correspondence of store depletion and the opening of the plasma membrane  $\text{Ca}^{2+}$  channels and the identity of the relevant channels was the subject of extensive research. Theories for the activation mechanism for SOCC included the diffusible messenger hypothesis in which an acid factor isolated from activated T lymphocytes, referred to as  $\text{Ca}^{2+}$  influx factor (CIF), showed the ability to activate  $\text{Ca}^{2+}$  influx for  $\text{Ca}^{2+}$  release activated  $\text{Ca}^{2+}$  currents (CRAC) (Randriamampita and Tsien 1993; Smani, Zakharov et al. 2003; Smani, Zakharov et al. 2004; Bolotina 2008). The conformational coupling hypothesis was taken from the excitation-contraction coupling mechanism in skeletal muscle, in which a plasma membrane dihydropyridine sensitive  $\text{Ca}^{2+}$  channel is coupled to a ryanodine receptor on the ER. The hypothesis for store operated entry was that the  $\text{IP}_3$  receptor in the ER was directly linked to a plasma membrane  $\text{Ca}^{2+}$  entry channel. Upon ER store depletion a conformational change in the  $\text{IP}_3\text{R}$  was proposed to signal opening of the SOCC (Berridge 1995). However, further studies showed that interaction with the  $\text{IP}_3\text{R}$  was not required, demonstrated

by inhibition of SOCC in IP<sub>3</sub>R knock out cell models (Prakriya and Lewis 2001; Sweeney, McDaniel et al. 2002). Establishing the identity of SOCC candidates still remained difficult, transient receptor potential (TRP) channels had been considered although they possess different pore properties to CRAC (Parekh and Putney 2005). Patch-clamp studies greatly contributed by characterising the store operated Ca<sup>2+</sup> current; the CRAC current (Parekh and Putney 2005; Prakriya 2009) initially in T lymphocytes and Jurkat leukemic T cells (Lewis and Cahalan 1989; Zweifach and Lewis 1993). Studies revealed that the CRAC channel is required for sustained Ca<sup>2+</sup> influx during antigenic stimulation of T cells (Partiseti, Le Deist et al. 1994; Feske, Giltman et al. 2001). It was also discovered that patients with severe combined immunodeficiency (SCID) had T cells that lacked SOCC and CRAC (Partiseti, Le Deist et al. 1994; Feske, Prakriya et al. 2005). Using large scale gene silencing through small interference RNA (siRNA) to target SOCC; in 2005, breakthrough studies identified stromal interaction molecule 1 (STIM1), a mammalian ER Ca<sup>2+</sup> sensor and conformational coupler between the ER and CRAC channels (Liou, Kim et al. 2005; Roos, DiGregorio et al. 2005), which worked through relocation towards the plasma membrane. By 2006, Orai1 was identified as a pore component of the mammalian CRAC channel (Feske, Gwack et al. 2006; Vig, 2006; Vig, Beck et al. 2006; Zhang, Yeromin et al. 2006) (figure 1.10).



**Figure 1.10. Structural features of STIM1 and Orai1 proteins.** STIM1 senses ER Ca<sup>2+</sup> depletion then translocates to sites adjacent to the PM to form puncta with Orai1, a plasma membrane (PM) pore-forming subunit of the CRAC channel.

Orai1 possesses four PM spanning regions, N and C termini. Critical residues are shown. Glutamates (yellow) and aspartates (light green) affect ion selectivity, permeation and blocking. A spontaneous mutation causes a loss of CRAC in patients with SCID (orange). The C terminal region required for activation from STIM1 is indicated (red).

STIM1 features an unpaired EF hand and sterile  $\alpha$  motif (SAM) domain on the ER luminal side. In the cytosolic region, coiled-coil (CC), ezrin-radixin-moesin (ERM), serine-proline-rich (S/P) and lysine-rich (K) domains are shown. Adapted from (Prakriya, Feske et al. 2006; Vig, Beck et al. 2006; Yeromin, Zhang et al. 2006; Lewis 2007; Feske, 2006).

### 1.2.3.i Stromal Interaction Molecule 1 (STIM1)

STIM1 was first identified as a cell surface transmembrane phosphoprotein characterised as a tumour growth suppressor (Parker, Begley et al. 1996; Manji, Parker et al. 2000). In a large scale RNAi screening in drosophila and parallel study in mammalian homologues targeting proteins with transmembrane domains, STIM1 proteins were found to be required for store operated entry and CRAC activation (Liou, Kim et al. 2005; Roos, DiGregorio et al. 2005). It was also noted that the

siRNA targeting STIM had no effect on intracellular  $\text{Ca}^{2+}$  stores or resting cytosolic  $\text{Ca}^{2+}$  concentration, despite the absence of store operated entry (Liou, Kim et al. 2005), (Roos, DiGregorio et al. 2005). Dr S. Peel (Therapeutic and Molecular Medicine, QMC, Nottingham) was the first to show the role of STIM1 in human airway myocytes, acting as a signal for SOCC activation following intracellular  $\text{Ca}^{2+}$  store depletion (Peel, Liu et al. 2006). STIM1 and its homologue STIM2 are type 1a single pass transmembrane proteins containing a coiled coil region, two N-linked glycosylation sites and feature a luminal helix-turn-helix EF hand  $\text{Ca}^{2+}$ -sensing module and a sterile alpha motif (SAM) (Ponting 1995; Williams, Manji et al. 2001; Williams, Senior et al. 2002). STIM1 is predominantly localised to the ER but is shown to form puncta, accumulating in subregions of junctional ER located 10-25nm from the PM just prior to CRAC opening (no STIM insertion into the PM was detected) (Williams, Manji et al. 2001; Liou, Kim et al. 2005; Zhang, Yu et al. 2005). Puncta formation is suggested to be the result of decreased binding of  $\text{Ca}^{2+}$  to the EF hand motif, causing the oligomerization of STIM1 leading to translocation of the multimers to corresponding PM adjacent sites for STIM1 initiated  $\text{Ca}^{2+}$  influx (Stathopoulos, Li et al. 2006; Liou, Fivaz et al. 2007). The aggregation of STIM1 is shown to induce clustering of Orai on the PM (Xu, Lu et al. 2006; Putney 2007; Varnai, Toth et al. 2007; Barr, Bernot et al. 2008).

### **1.2.3.ii Orai1**

Orai1 was discovered through genetic linkage studies from mutations in a rare form of severe combined immunodeficiency (SCID) in which T cell store depletion failed to activate  $\text{Ca}^{2+}$  entry (Feske, Prakriya et al. 2005), showing an

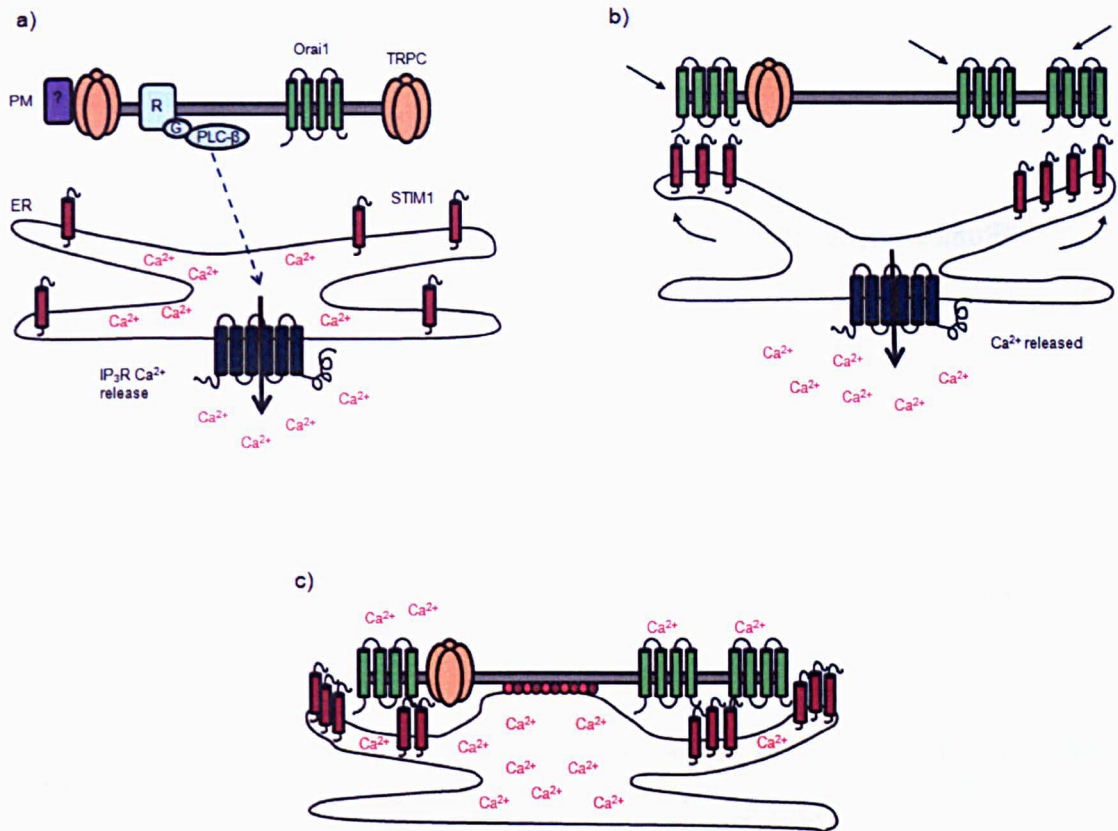


absence of CRAC activity (Feske, Gwack et al. 2006). Linkage analysis and positional cloning identified a region of interest on chromosome 12 (Feske, Gwack et al. 2006), further genome wide RNAi screening in S2 cells led to identification of a previously uncharacterized gene, given the name Orai (or CRACM1) (Vig, Beck et al. 2006; Zhang, Yeromin et al. 2006). Orai1 and its family members Orai2 and Orai3 are proposed to be 4 transmembrane domain, plasma membrane spanning proteins (Feske, Gwack et al. 2006). The main roles of Orai2 and Orai3 still remain unclear. Studies show that expression of Orai1 alone reduces SOCC, however coexpression with STIM1 indicates a large  $\text{Ca}^{2+}$  influx or amplification of CRAC (Mercer, Dehaven et al. 2006; Peinelt, Vig et al. 2006; Soboloff, Spassova et al. 2006). Similar properties were shown for Orai2 but not Orai3 (Mercer, Dehaven et al. 2006). Dr S. Peel (Therapeutics and Molecular Medicine) had previously shown that STIM1 plays a role in SOCC activation following store depletion in HASM and set out to investigate whether Orai plays a role in this pathway of  $\text{Ca}^{2+}$  influx (Peel, Liu et al. 2008). Together results implicate both STIM1 and Orai as potential modulators of  $\text{Ca}^{2+}$  influx through SOCC in HASM.

### **1.2.3.iii STIM - Orai interaction**

Store depletion is sensed by STIM1, causing it to cluster in ER sites adjacent to the PM. In resting cells Orai1 proteins show even PM distribution, however, upon store depletion Orai1 rapidly clusters to co-localize with STIM1 forming puncta (Luik, Wu et al. 2006; Xu, Lu et al. 2006; Varnai, Toth et al. 2007), shown using TIRF-based  $\text{Ca}^{2+}$  imaging. Confusion still remains over whether there is direct interaction between STIM and Orai. The STIM proteins C-terminal polybasic

domain was suggested to facilitate interaction with the PM (Liou, Fivaz et al. 2007), however, studies showed that despite a deletion in the polybasic domain, CRAC activation was possible (Spassova, Soboloff et al. 2006; Li, Lu et al. 2007). There is also a suggestion that Orai1 may associate with a larger protein complex within the PM allowing for a multi-protein site for interaction between STIM1 and Orai1, directly or indirectly (Varnai, Toth et al. 2007). In addition, TRPC channels have been shown to mediate receptor activated  $\text{Ca}^{2+}$  influx (Venkatachalam and Montell 2007). Electrostatic interaction studies indicate that STIM1's polybasic tail is required to gate TRPC1 and TRPC3 by a similar mechanism. However, the polybasic and S/P domains of STIM1 are not required for activation of Orai1 (Zeng, Yuan et al. 2008). Studies have also demonstrated an interaction between Orai and TRPC supporting the possibility that TRPC channels play a role in SOCC (Cheng, Liu et al. 2008; Liao, Erxleben et al. 2008). Despite advancements the process for relocation, interaction and CRAC channel opening still remains unclear (relocation and formation of puncta for STIM1 and Orai1 is shown in figure 1.11).



**Figure 1.11. Current model for Store Operated Calcium Channel (SOCC) entry.** a) STIM1 and Orai1 are dispersed throughout the endoplasmic reticulum (ER) and plasma membrane (PM), agonist stimulation of a G-protein coupled receptor (R) acting through the PLC- $\beta$  pathway leads to ER stored  $\text{Ca}^{2+}$  release via the IP<sub>3</sub>R. A TRPC complex is also present at the PM. b) ER located STIM1 senses this store depletion and relocates to cluster at sites adjacent to the PM. Simultaneously Orai1 relocates in regions of the PM directly opposite the STIM1 clusters. c) this interaction allows  $\text{Ca}^{2+}$  influx for store refilling. Adapted from (Luik, Wu et al. 2006; Lewis 2007; Luik and Lewis 2007).

The ability to manipulate the entry process has proven difficult due to the lack of available specific SOCC inhibitors (Putney 2001) (discussed in 3.4.8). Trivalent cations such as lanthanum ( $\text{La}^{3+}$ ) ( $\text{IC}_{50} = 10 - 100 \text{ mM}$ ) and gadolinium ( $\text{Gd}^{3+}$ ) ( $\text{IC}_{50} < 1.0 \text{ mM}$ ) still remain the most used inhibitors of SOCC in many cell types (Luo, Broad et al. 2001). However, these ions inhibit a wide range of  $\text{Ca}^{2+}$  entry channels (Trebak, Bird et al. 2002). As mentioned previously,  $\text{Ca}^{2+}$  is taken up from the cytosol into the internal stores by the sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$ -

ATPase (SERCA) pump.  $\text{Ca}^{2+}$  entry can also be activated by inhibition of the SERCA pumps using cyclopiazonic acid and thapsigargin (Thastrup, Cullen et al. 1990); showing that influx can be stimulated without the phospholipase C pathway (Putney 2007). In addition,  $\text{Ca}^{2+}$  chelators such as ethylene glycol tetraacetic acid (EGTA), can be used experimentally to bind extracellular  $\text{Ca}^{2+}$ , reducing  $\text{Ca}^{2+}$  availability for influx and store refilling (Smyth, Dehaven et al. 2006).

#### **1.2.4 $\text{Ca}^{2+}$ sensitivity through RhoA-Rho Kinase interactions**

ASM contraction is dependent on phosphorylation by MLCK and relaxation is dependent on dephosphorylation by MLCP. It is currently hypothesised that MLCP is regulated by RhoA kinase phosphorylation of the MYPT1 subunit, protein kinase C (PKC) and the MLCP inhibitor CPI-17; this inhibition of MLCP activity increases  $\text{Ca}^{2+}$  sensitivity (Somlyo and Somlyo 2003). A ligand binds the G protein coupled receptor which activates tyrosine kinase, this initiates a signalling cascade to activate RhoA to translocate to the membrane. RhoA then activates Rho kinase, which in turn phosphorylates MLCP, this causes inactivation of MLCP, leading to a net accumulation of phosphorylated myosin light chains, resulting in increased contraction (Janssen and Killian 2006). The RhoA-Rho kinase (ROCK) pathway therefore contributes to cholinergic stimulated sustained contraction. In addition, agonists increase  $\text{Ca}^{2+}$  sensitivity through activation of the G protein which stimulates Rho kinase, to activate PKC via DAG (Parekh, Ziegler et al. 2000). ROCK inhibitors have shown to be effective bronchodilators (Nakahara, Moriuchi et al. 2000; Liu, Zuo et al. 2006). RhoA/ ROCK pathway members are also shown to be increased in allergic animal models (Chiba, Takada et al. 1999; Chiba, Sakai et al.

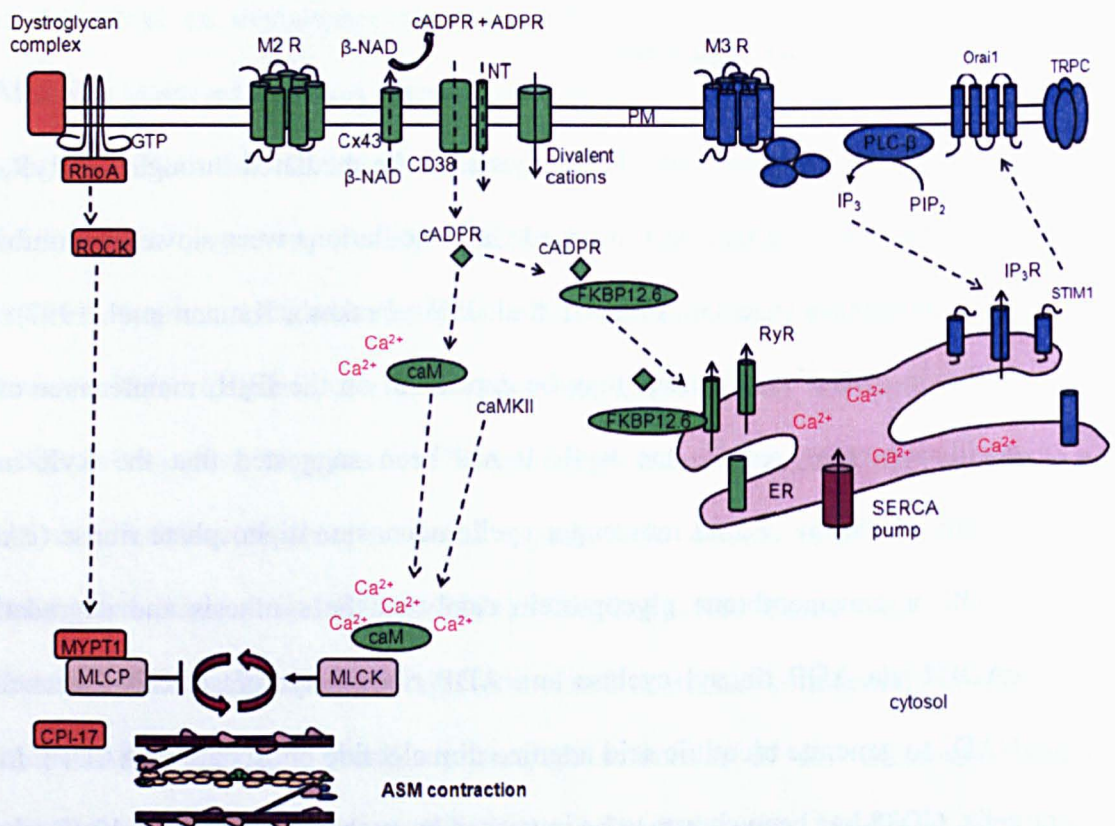
2001; Hashimoto, Nakano et al. 2002; Chiba, Sakai et al. 2003; Chiba and Misawa 2004; Sakai, Ootogoto et al. 2004). Studies have linked the RhoA-Rho kinase pathway with hyper-responsiveness through experiments involving cytokines (Parris, Cobban et al. 1999; Hunter, Cobban et al. 2003) and mechanical stress (Smith, Roy et al. 2003). Telokin, a 17-KDa protein specific for smooth muscle is phosphorylated by cAMP and cGMP and reportedly promotes  $\text{Ca}^{2+}$  desensitisation via activation of MLCP in intestinal smooth muscle (Wu, Haystead et al. 1998; Choudhury, Khromov et al. 2004). Its presence and role in ASM are unknown.

### **1.2.5 Ryanodine receptor (RyR) pathway involved in $\text{Ca}^{2+}$ oscillations and internal $\text{Ca}^{2+}$ store regulation**

$\text{Ca}^{2+}$  oscillations are also suggested to be mediated through the RyR, based on studies showing that ACh induced  $\text{Ca}^{2+}$  oscillations were slowed or inhibited by RyR antagonists (Kannan, Prakash et al. 1997; Prakash, Kannan et al. 1997). While initiation of  $\text{Ca}^{2+}$  oscillations may be dependent on the  $\text{IP}_3\text{R}$ , maintenance of these oscillations may require the RyR. It has been suggested that the RyR may be sensitised via the second messenger cyclic adenosine diphosphate ribose (cADPR). CD38, a transmembrane glycoprotein catalyzes the synthesis and degradation of cADPR via ADP ribosyl cyclase and ADP ribosyl hydrolase. CD38 also uses  $\beta\text{-NAD}^+$  to generate nicotinic acid adenine dinucleotide phosphate (NAADP). In ASM cells, CD38 has been shown to be increased by such cytokines as IL-13 (Deshpande, Dogan et al. 2004),  $\text{TNF-}\alpha$  (Deshpande, Walseth et al. 2003) and IL-1 $\beta$ . cADPR mediated  $\text{Ca}^{2+}$  release may involve activation of the RyR2, the predominant isoform in ASM (Kannan, Prakash et al. 1997). Activation of RyR may occur indirectly via



such accessory proteins as FKBP 12.6 which maintains RyR2 in an inactive state (Berridge, Bootman et al. 2003). Studies suggest that increased cADPR could lead to increased  $\text{Ca}^{2+}$  responses. Addition of extracellular cADPR potentiates ACh induced contractions in bovine ASM (Franco, Bruzzone et al. 2001). Treatment of HASM with IL-13 increases CD38 expression and cADPR cyclase activity, cADPR antagonists have also been shown to abolish the effects of TNF- $\alpha$  and IL-13 on an agonist induced  $\text{Ca}^{2+}$  response (Deshpande, Dogan et al. 2004; Deshpande, Walseth et al. 2003). FKBP KO mice have also shown increased contractile responses to cholinergic stimulation (Wang, Zheng et al. 2004).



**Figure 1.12.** Summary of pathways involving store operated  $\text{Ca}^{2+}$  entry (blue), internal  $\text{Ca}^{2+}$  store regulation (green) and  $\text{Ca}^{2+}$  sensitivity (orange). Agonist activation leads to stimulation of the  $\text{IP}_3$  pathway (blue), activating release of  $\text{Ca}^{2+}$  from the sarcoplasmic /endoplasmic reticulum (ER) via the  $\text{IP}_3\text{R}$ . The depletion of internal stored  $\text{Ca}^{2+}$  results in dissociation of  $\text{Ca}^{2+}$  from STIM1 in a

subcompartment of the ER, which causes STIM1 to relocate within the ER to areas near Orail channels located in the plasma membrane (PM); to activate Orail channels for  $\text{Ca}^{2+}$  influx. The pathway in green shows activation of the M2 subtype muscarinic receptor, which is coupled to an  $\alpha_i$  G-protein. This recruits the CD38 signalling pathway.  $\beta\text{NAD}$  is transported out of the cytoplasm by connexin 43 hemichannel proteins (Cx43). cyclic adenosine diphosphate ribose (cADPR) is generated by CD38 and transported back into the cytoplasm either through nucleoside transporters (NT) or via CD38. ADPR, a product of the CD38 enzymatic activity acts on transient receptor potential (TRP) channels to allow influx of divalent cations, including  $\text{Ca}^{2+}$ . cADPR acts directly or indirectly with the ryanodine receptor (RyR) to open the ion channel. FK506-binding protein 12.6 (FKBP12.6) is associated with the RyR and keeps it in a closed state. Interaction of cADPR with the RyR causes disassociation of FKBP12.6 and increases the open probability of the receptor to release sarcoplasmic reticulum  $\text{Ca}^{2+}$ . Calmodulin (CaM) is shown to play a role in cADPR mediated events in some species, through recruitment of CaM kinase II (CaMKII) to phosphorylate the RyR, this phosphorylation also increases the open probability of the RyR. Finally, cADPR acts directly on the RyR channel to open it. The pathway in orange depicts  $\text{Ca}^{2+}$  sensitisation. Studies indicate that  $\text{Ca}^{2+}$  sensitisation is linked to decreased MLCP activity; through phosphorylation and inhibition of the regulatory myosin phosphatase subunit (MYPT1) of MLCP by Rho kinase and by phosphorylation of CPI-17, an inhibitory protein of type 1 protein phosphatase, by protein kinase C (PKC). The production of Rho involving diacylglycerol (DAG) to active Rho kinase and PKC are processes that involve agonist activation of the G-protein coupled receptors, however, depolarisation by KCl has also lead to  $\text{Ca}^{2+}$  sensitivity in vascular smooth muscle. Diagram adapted from (Parekh, Ziegler et al. 2000; Ratz, Berg et al. 2005; Smyth, Dehaven et al. 2006; Putney 2007; Sanderson, Delmotte et al. 2008).

## 1.3 DEVELOPING A MODEL TO STUDY AIRWAY SMOOTH MUSCLE CONTRACTION IN DISTAL AIRWAYS

### 1.3.1 The distal airways

*In vivo*, the influence of mechanical and physiological stresses during normal respiratory function on airway narrowing at varying generations of the bronchial tree have been difficult to study. Histological studies suggest the large airways as the site of greatest narrowing (Okazawa, Vedal et al. 1995), however imaging of isolated airways and lung preparations suggest the smaller and mid-sized airways show the greatest degree of narrowing (Amirav, Kramer et al. 1993; Mitchell, Cvetkovski et al. 1998; Bai, Zhang et al. 2007). Despite the amount of smooth muscle, cartilage thickness and number of mucosal folds being greater in large airways; porcine studies have demonstrated that increased sensitivity to ACh, muscle shortening and degree and rate of luminal narrowing are shown to be greater in small airways than in larger airways (Noble, McLaughlin et al. 2010; Noble, West et al. 2010; Mitchell, Cvetkovski et al. 1998); possibly due to cartilage limiting contraction in larger airways. Taken together the majority of the evidence suggests the distal airways exhibit increased sensitivity, rate and degree of narrowing in comparison to proximal airways.

In human, the airways are divided into 24 generations; the distal airways are classified as < 2mm internal diameter in adults, non-cartilaginous and spanning the 8<sup>th</sup> generation down to terminal bronchioles and respiratory bronchioles (Burgel, Frachon et al. 2009). Studies of distal airways have proved difficult due to the limited techniques available. In asthmatic patients distal airways potentially contribute to up to 90% of airflow resistance (Van Brabant, Cauberghs et al. 1983;



Yanai, Sekizawa et al. 1992); suggesting this is the main site of airflow obstruction (Ingram 1990; Ohrui, Sekizawa et al. 1992; Kuwano, Bosken et al. 1993; Wagner, Bleecker et al. 1998) and AHR (Wiggs, Bosken et al. 1992; Kuwano, Bosken et al. 1993). Similar yet more severe inflammatory and structural changes occur in the distal lung and lung parenchyma of asthmatics compared to proximal sites (Carroll, Carello et al. 1996; Kraft, Djukanovic et al. 1996; Faul, Tormey et al. 1997). Regional variations in inflammatory cell distribution in asthmatics are also suggested, with distal airways predominantly showing CD45+ leukocytes and eosinophils in the outer airway wall regions (between the smooth muscle and alveolar attachments), in comparison large asthmatic airways showed a greater density of eosinophils in the inner airway wall regions, between the basement membrane and smooth muscle (Haley, Sunday et al. 1998). These differences in regional distribution of inflammatory cells may reflect different mechanisms of inflammatory cell recruitment and chemokine/cytokine production (Hamid and Tulic 2007).

While airway inflammation occurs throughout the airway generations, given the recent evidence highlighting the significance of the small airways in inflammation and airflow resistance in asthmatic patients; this raises concern over the efficiencies of delivery of current treatments. Inhaled corticoids reduce airway inflammation in mild-moderate asthmatics (Djukanovic, Wilson et al. 1992; Booth, Richmond et al. 1995); however fail to relieve airway inflammation and hyper-responsiveness in severe asthmatics despite long term treatment (Jeffery, Godfrey et al. 1992; Gauvreau, Inman et al. 2002). Current therapy is largely based on inhaled drugs with a particle size of 3 - 5  $\mu\text{m}$  (Burgel, Frachon et al. 2009). Deposition studies predict that deposition occurs predominantly in the proximal airways

(Esmailpour, Hogger et al. 1997). Strategies for targeting the distal airways include intravenous administration or inhaled medications using extra-fine particles to deposit in the proximal and distal airways. A few studies have trialled delivery of extra fine particles via metered dose inhaler (MDI); e.g a study quantifying lung and extra thoracic aerosol deposition of 1.5, 3 and 6  $\mu\text{m}$  diameter particles found the smaller particles achieved greater total and peripheral lung deposition (Usmani, Biddiscombe et al. 2005). Use of hydrofluoroalkane-beclomethasone dipropionate (HFA-BDP) pressurised metered-dose inhaler extra-fine aerosol suggests improved targeting compared to the chlorinated fluorocarbon CFC (chlorofluorocarbon) propellants (Leach, Davidson et al. 1998; Busse, Brazinsky et al. 1999; Goldin, Tashkin et al. 1999; Hauber, Gotfried et al. 2003).

### **1.3.2 Developing a physiologically relevant model for studying airway smooth muscle contraction in the distal airways.**

To explore the mechanisms involved in ASM contraction and the pathogenesis of hyper-reactivity implicated in asthma, previous studies have generally used lung function tests in whole airways. Given that the air to water interfaces within the lung create difficulty for imaging methods; most understanding of lung function in asthmatics is derived from measurements of spirometry and plethysmography, predominantly reflecting large airway contribution. Previously mentioned, measurement of lung function and airway obstruction in humans can be achieved through non invasive tests designed to measure forced expiration; from this, values for peak expiratory flow (PEF), forced expiratory volume in one second ( $\text{FEV}_1$ ) and MMEF (maximal midexpiratory flow) can be calculated (Dawson and Elliott 1977).

Recently refined imaging techniques such as CT have also been used to measure the airway structure within the lung (Amirav, Kramer et al. 1993).

A range of *ex vivo* and *in vivo* models have been used to study airway function, these are summarised in table 1.3. Measurement of expiratory flow rate in animal models is technically challenging, therefore methods to assess mechanical response have been designed. Trapped gas volume involves measuring the volume of gas in the lungs dissected from animals (Yiamouyiannis, Stengel et al. 1995), based on the theory that in induced airway constriction a greater proportion of airways are closed near functional residual volume (FRC). Animal models that require anaesthesia and instrumentation are used for airway pressure measurements during mechanical ventilation, by inserting a tracheal cannula with a side tap for measuring pressure (Levitt and Mitzner 1988). Anaesthesia and invasive instrumentation is also required for measuring pulmonary resistance and lung compliance (Drazen, Finn et al. 1999). The disadvantage of using anaesthetic agents is that they may cause agonist or antagonistic effects on some receptors, or alter neural response and lung blood flow.

Contraction of isolated airway segments is a popular method for studying airway contraction and involves dissection of airways for organ bath. By attaching the tissue to force or motion transducers, either isotonic or isometric responses can be measured. However, this method of tissue dissection disrupts the neural and circulatory pathways which may alter pharmacological response (Larsen, Renz et al. 1992). In addition, in general measurements are based on stretching of isolated muscle strips rather than assessment of cross sectional area of an intact airway.

Models of mechanical response provide information about airway contraction, but not the underlying process. Use of isolated smooth muscle cells (ISMC) provides

insight into some processes of  $\text{Ca}^{2+}$  signalling, however the contraction cannot be observed and there is an argument that in culture the cells dedifferentiate and the contractile machinery is lost or significantly reduced (Widdop, Daykin et al. 1993). Such difficulties highlight the need for an intact airway model that allows simultaneous quantification of contraction and measurement of relevant signalling pathways.

**Table 1.3. Common techniques used to assess airway contraction in human and animal models; a comparison of the advantages and disadvantages.**

Model	Techniques	Advantages	Disadvantages	Reference
<b>Human</b>	Isolated smooth muscle cells	Provides information about the cellular processes involved in contraction, non invasive.	Cells may dedifferentiate in culture, contractility may be lost. Does not show contractile effects of signalling.	(Widdop, Daykin et al. 1993; Bergner and Sanderson 2002)
	Lung function tests in whole airways	Non invasive, can be calculated from minimal expiratory effort.	Technically difficult to measure in animal models. No regional information.	(Dawson and Elliott 1977; Drazen, Finn et al. 1999)
<b>Animal</b>	<b>Anaesthesia and invasive techniques</b>	Intact anatomical structures in animal, meaning contraction can be influenced by neural and inflammatory mediators.	Anaesthesia may alter neural pathways, vasodilatation and receptor behaviour.	(Yamakage 2002; Yamakage and Namiki 2003)
	Airway pressure measurements during mechanical ventilation	Simple procedure to measure pressure.	Measurements do not differentiate between changes in airway resistance or in lung elasticity.	(Levitt and Mitzner 1988; Drazen, Finn et al. 1999)
	Measurement of pulmonary resistance and lung compliance	Breath-by-breath measurements for pulmonary resistance and dynamic pulmonary compliance can be given; meaning changes due to agonists can be accurately assessed.	Technically difficult, invasive instrumentation in intact animals required. Requires multiple measurement variables to calculate transpulmonary pressure.	(Martin, Gerard et al. 1988)
	<b>Techniques requiring lung removal</b>	Technically easy to dissect.	Receptor stimulation, innervations and circulation may be altered with tissues dissected free of body; which may affect contractile response.	(Drazen, Finn et al. 1999)
	Contraction of	Multiple experiments and tissue from one animal,	Innervation and circulation of the tissue	(Larsen, Renz et

	isolated tissue segments	contraction measured directly, ability to control initial tissue segment length and tension.	segments is disrupted due to the tissue segments being dissected, which may alter contraction.	al. 1992; Manzini 1992)
	Trapped gas volume	Technically easy to produce a measurement of trapped gas volume, all lung structures are intact	Only one measurement is given for each animal, relevance of trapped gas volume measurement to airway obstruction is unknown.	(Yiamouyiannis, Stengel et al. 1995; Drazen, Finn et al. 1999)
	Mechanical measurements of dissected lungs	Static compliance and pulmonary resistance can be measured	Static pulmonary compliance measurements reflect mechanisms of the lung parenchyma rather than the airways, therefore there is little relevance to asthma models.	(Hirai, Hosokawa et al. 1995)
<b>Human and animal</b>	Precision cut lung slices (PCLS)	One animal can be used for multiple experiments. Applied to diverse range of techniques: measurements of $[Ca^{2+}]_i$ , siRNA gene silencing and pharmacological methods to study influences on contraction/ signalling pathways.	Innervation and circulation of the tissue segments is disrupted. Preparation of lungs for slicing may alter integrity of airways thus effecting contractile response.	(Bergner and Sanderson 2002)

## 1.4 AIMS

Much of the work described in this thesis utilises Precision Cut Lung Slices (PCLS), allowing the study of distal airway behaviour. By preparing slices using a vibrating microtome tissue slicer, this method generates multiple experiments using small amounts of lung tissue. In addition to measuring contraction, PCLS can also be used to study other responses such as  $\text{Ca}^{2+}$  signalling in ASM using confocal microscopy (Bergner and Sanderson 2002). Through knock out mouse models and manipulation of the inflammatory environment, this lung slice technique allows insight into the pathogenesis of the airway inflammatory response and airway hyper-reactivity at the distal airway level in a range of models of asthma.

The aims of the studies described in this thesis were:

- To characterise the contractile response of PCLS in wild type mice and pigs to serve as a prelude to future studies using human lung tissue.
- To investigate the role of  $\text{Ca}^{2+}$  in ASM contraction and how dysregulation of  $\text{Ca}^{2+}$  homeostasis may contribute to AHR in patients with asthma.
- To use the PCLS technique to characterise airway responses in murine models of allergic sensitisation designed to mimick specific features of the  $\text{T}_\text{H}2$  inflammatory response seen in human patients with asthma.
- To define key inflammatory mediators influencing ASM contractile responses and to explore the pathway required for AHR in these models; also to investigate whether these mediators may contribute to AHR in human patients with asthma.

## **CHAPTER TWO**

### **MATERIALS & METHODS**



## **2. CHAPTER TWO**

### **2.1 ANIMAL TISSUE LUNG SLICE PREPARATIONS AND CONTRACTION EXPERIMENTS**

The precision cut lung slice (PCLS) technique was developed by Bergner and Sanderson (2002, University of Massachusetts, Worcester USA) for use on mouse lung slices. The slices are prepared by inflating the lung lobes with agarose and cutting the solidified lung tissue using an oscillating tissue slicer to obtain slices of < 200  $\mu\text{m}$  thickness. These slices contain airways that remain structurally intact with visibly beating cilia on the epithelia lining. This model provides a physiologically relevant system to study airway contraction. The contraction experiments described in this thesis required real time video caption using bright field settings, imaging software was then used to measure the airway lumen area at selected time points throughout the experiments.

#### **Materials for preparation of lung slices**

- HBSS buffer: HEPES minimum 99.5% titration 10 mM (H3375-5009, Sigma, Germany), magnesium chloride 1 mM (M2670-5009, Sigma, Germany), calcium chloride 2 mM (Code c/1500, FSA Laboratory Supplies, Loughborough, UK). Hank's Balanced Salt Solution (HBSS) (without  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , Ph red (10x) (CE) 500 ml cat. no. 14185, Invitrogen Ltd, UK).
- DMEM D5796 (Sigma-Aldrich Inc., St Louis, Mo., USA), supplemented with 2 mM glutamine, Penstrep (2.4 ml/l, Penicillin 10.000 U/ml +

Streptomycin 10.000 µg/ml) and Fungizone (4.8 ml/l, Amphotericin B 250 UG/ml).

- Agarose Type IV low gelling (A9045-25G, cas. 39346-81-1, Sigma-Aldrich Inc., St Louis, Mo., USA).
- Catheters: BD Saf-T-Intima 20 GA 1.00 IN 1.1 x 25 mm 55 ml/min (Beckton Dickinson cat. no. 383335, Denmark).
- Quick Bond tissue adhesive glue (Type 221 AA.471, cat. no. 72588, Science services, UK). Sectioning blades (for EMS tissue slicer) (cat. no. 93020, Science services, Munich, Germany).
- Oscillating Tissue Slicer EMS 5000 (Serial number 16234, Electron Microscopy Sciences, P.O. Box 550, Hatfield Pa 19440) and Bath medium refrigeration controller for EMS 5000 (<http://www.emsdiasum.com>). Power Cable for 93220 Tissue Slicer (E93220-pack, Science services, UK). Fuses (stock no. 563 633A BP, 500 milliamp, 5 mm x 20 mm anti surge, RS components, UK).

### **2.1.1 Mouse Lung Slices**

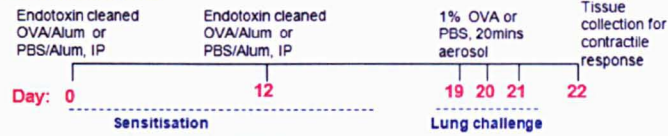
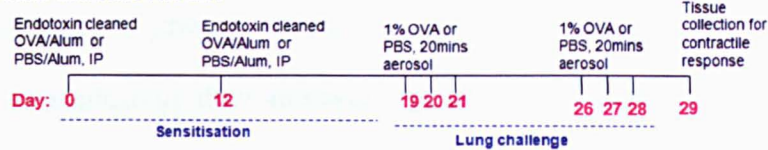
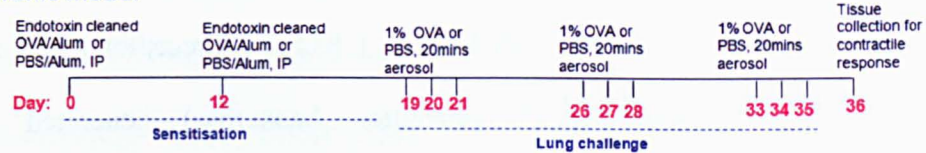
*Mice for Chapter Three:* were taken from normal female, five - ten week old, C57BL/6 mice. These mice were supplied by Dr Luisa Martinez-Pomares (Immunology, QMC, Nottingham). These mice were also used for an experiment in Results Chapter Four, as stated.

*Mice for Chapter Four: IL-33 as a key mediator of airway hyper-responsiveness in a murine model of airway inflammation.* Female BALB/c mice were obtained from

Charles River. All mice were housed in the Medical Research Council Laboratory of Molecular Biology, Cambridge (McKenzie group). Mice were housed in pathogen free, environmentally controlled conditions and were six - twelve weeks old when euthanized. All animal experiments were undertaken with the approval of the UK Home Office. For ovalbumin sensitisation results shown, mice underwent acute, mid-chronic and chronic ovalbumin sensitisation with phosphate buffered saline solution (PBS) matched controls (described below). Naïve WT mouse lung slices (130  $\mu\text{m}$  thickness) were also incubated overnight in selected cytokines (100 ng/ml). Lungs were also obtained from IL-1RL-1 KO (ST2 receptor knockout) mice on a BALB/c background (sixth generation backcross) generated as described (Townsend, Fallon et al. 2000) and the combination of IL-4, IL-5, IL-9 & IL-13 KO mice generated as described previously (Fallon, 2002).

#### **2.1.1.i Protocols for acute, mid-chronic and chronic ovalbumin sensitisation in BALB/c mice (allergic airway disease model)**

Sensitisation was performed by the McKenzie group at Cambridge. Mice were sensitised by intraperitoneal administration of endotoxin-low ovalbumin (OVA) on day zero and day twelve (20  $\mu\text{g}$ / dose in 50  $\mu\text{l}$  of PBS mixed with 50  $\mu\text{l}$  of aluminium hydroxide (alum)). Control animals received PBS. Mice were challenged with aerosolised 1% (v/v) OVA for 20 minutes in a custom made air tight gas chamber on day 19, 20 and 21. This three-day challenge protocol was repeated every seven days for up to three weeks. Lung tissue was taken 24 hours after the final aerosol treatment, either on day 22, 29, or 36 (see below).

**Acute model****Mid-chronic model****Chronic model**

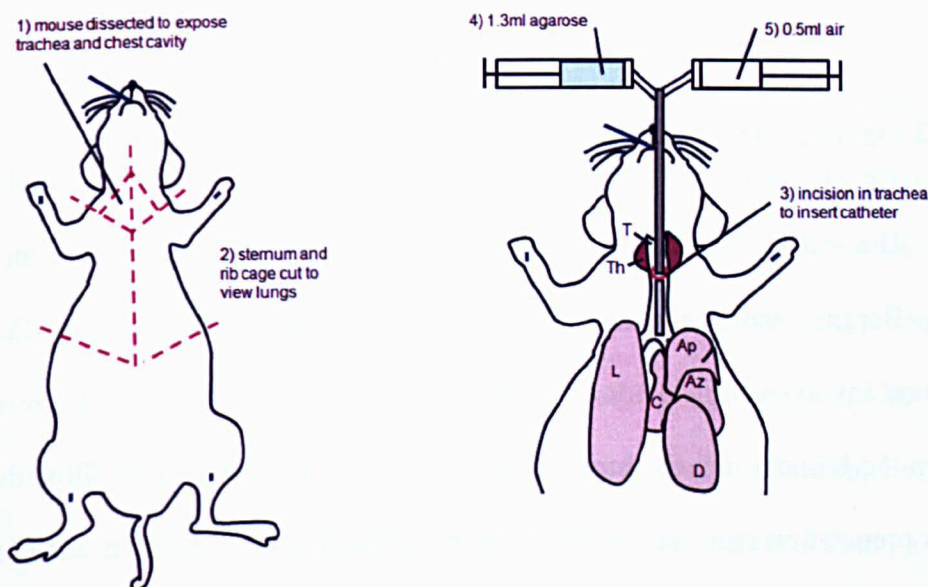
**Figure 2.1. Protocols for acute, mid-chronic and chronic ovalbumin sensitisation** (described above). OVA: ovalbumin; PBS: phosphate buffered saline solution; Alum: aluminium hydroxide; IP: intraperitoneal injection.

### 2.1.1.ii Method for preparation of mouse lung slices:

1. Prior to experiments 1 liter of HBSS buffer was made up: sterile distilled H<sub>2</sub>O to 1L final volume; 10 mM (2.38 g) HEPES minimum 99.5% titration; 1 mM (1 ml of 1 M stock) magnesium chloride; 2 mM (2 ml of 1M stock) calcium chloride; 100 ml Hank's Balanced Salt Solution (HBSS) 10X; pH to 7.4 using NaOH.
2. Prior to the experiments 2% (w/v) low gelling temperature agarose was made up in HBSS buffer and stored in a water bath at 37°C for 30 minutes.
3. Mice were euthanized by intraperitoneal injection with 0.3 ml pentobarbitone sodium (Euthatol) followed by gentle neck dislocation. The mouse was opened from pharynx to diaphragm. The chest cavity was exposed by cutting along the sternum. A small hole was made midway along the trachea using a scalpel blade, a 20G intima, Beckton Dickinson catheter was then inserted into the trachea.

Attaching a 2 ml syringe (2 ml, Sabre, UK), the lungs were gently inflated with air to ensure the catheter was in the correct location and all lung lobes inflated, the lungs were then slowly deflated, string was tightened around the trachea to hold the catheter in place.

4. The 2% (w/v) low gelling agarose was slowly injected ( $\sim 1.3$  ml for eight - ten week mice) into the lungs until firm and 90% inflated, a syringe filled with air ( $\sim 0.5$  ml) to ensure airways would not be filled with agarose. The catheter was clamped to maintain pressure. The inflated lungs were covered with HBSS soaked cotton wool and left for 20 minutes, to allow the agarose to solidify before withdrawing the catheter and placing the lungs in HBSS buffer in a universal tube on ice. The lungs were then transported to the oscillating tissue slicer to be cut (see figure 2.2).



**Figure 2.2 Preparation of mouse lung slices** (described above). T: trachea; Th: thyroids; L: left lobe; Ap: apical lobe; Az: azygous lobe; D: diaphragmatic lobe; C: cardiac lobe.

### **2.1.1.iii Overnight cytokine incubation of BALB/c and c57BL/6 WT mouse lung slices**

For cytokine sensitisation experiments, naïve WT mouse lung slices were incubated overnight in 100 ng/ml of recombinant mouse IL-4, IL-5, IL-13, IL-24, IL-17A, IL-33 and TNF- $\alpha$  (R & D Systems, UK). Using a 96 well round bottomed plate (cat no. 163320, NUNc, Denmark), leaving the outer rows/columns empty (risk of evaporation), 150  $\mu$ l of DMEM media supplemented with Penstrep (2.4 ml/l, Penicillin 10.000 U/ml + Streptomycin 10.000  $\mu$ g/ml) and Fungizone (4.8 ml/l, Amphotericin B 250 UG/ml) was pipetted into each well. The cytokines were made up in sterile PBS to give stock concentrations (10  $\mu$ g/ml or 20  $\mu$ g/ml as specified). From this stock, cytokine concentrations were made up to 400 ng/ml in media, 50  $\mu$ l was then added to each well of the round bottomed plate for a 1:4 dilution. Two slices were placed into each well and left overnight at 37°C in 5% CO<sub>2</sub> for contraction experiments the following day.

### **2.1.2 Pig lung slices**

The method for preparation of precision cut lung slices for mouse lungs, taken from Bergner and Sanderson (2002), was adapted to obtain pig lung slices. This method involves cutting into the trachea and positioning tubing ( $\leq$  1 mm diameter) down the bronchi into the lung lobes to access a portion of lobe to fill with agarose. This preparation required the tubing to be positioned far enough into the lung lobes to be airtight, if not it was difficult to inflate. If only a section of lobe was obtained,



inflation was attempted by inserting a catheter or tubing into an airway and trying to isolate the section or block off other airways using the tubing and suture thread.

*Pigs for Chapter Four Results:* Domestic healthy pig whole lungs and trachea were obtained from Dr Richard Roberts (E34, QMC, University of Nottingham), freshly acquired from the Nottingham abattoir.

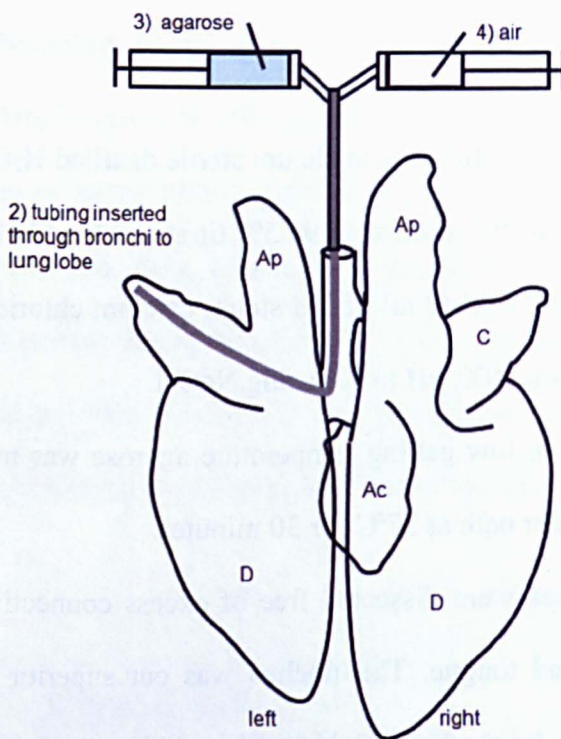
**Method for preparation of pig lung slices:**

1. Prior to experiments 1 L of HBSS buffer was made up: sterile distilled H<sub>2</sub>O to 1L final volume; 10 mM (2.38 g) HEPES minimum 99.5% titration; 1 mM (1 ml of 1M stock) magnesium chloride; 2 mM (2 ml of 1M stock) calcium chloride; 100 ml Hank's Balanced Salt Solution 10X; pH to 7.4 using NaOH.
2. Prior to the experiment 2% (w/v) low gelling temperature agarose was made in HBSS buffer and stored in a water bath at 37°C for 30 minutes.
3. The pig whole lungs and trachea were dissected free of excess connective and fatty tissue, the oesophagus and tongue. The trachea was cut superior to the bifurcation of the bronchus, at a level where the bronchi could be seen branching off. Tubing was connected to a 50 ml syringe via a three-way connection valve, this tubing was then inserted down the trachea into a bronchi and pushed through until a lobe was potentially located to be filled. Using the syringe this lobe was then filled with air to check for full inflation and slowly deflated again.
4. The low gelling temperature agarose (2% (w/v)) was pumped through the tubing to fill up the portion of lobe until it was 90% full and solid to touch, air was then pushed in (to clear the airways of agarose) (refer to figure 2.3). The tubing was

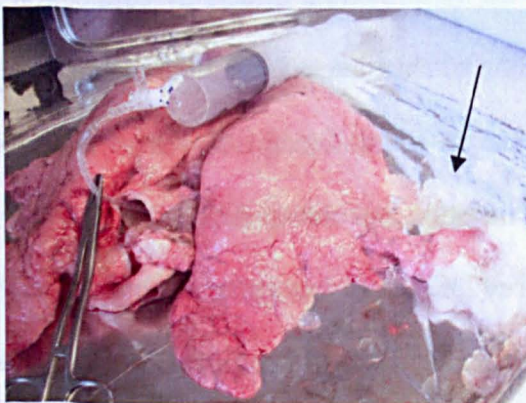


clamped in place and the tissue was left with cold HBSS soaked cotton wool covering it for 20 minutes until the agarose solidified completely, this portion of lung was then cut free and placed in a pot of HBSS on ice, to be cut using the oscillating tissue slicer.

1) pig lung set dissected free of muscle, connective tissue and oesophagus



**Figure 2.3. Preparation of pig lung slices.** Process for inflation of pig lungs with agarose followed by air to clear airways free of agarose leaving surrounding tissue to solidify. Ap: apical lobes; C: cardiac lobes; D: diaphragmatic lobes; Ac: accessory lobe.

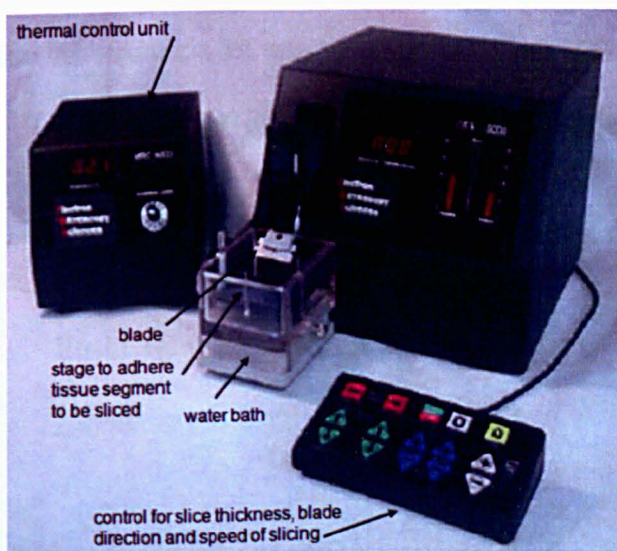


**Figure 2.4. Preparation of pig lung slices.** Tubing was inserted into the trachea, the agarose filled lobe is indicated.

Waste disposal and decontamination procedures: all dissection instruments were cleaned in 70% (v/v) ethanol, all syringes and catheters were disposed of in clinical waste. Tissue was double bagged in clinical waste bags, labeled and disposed of via off site incineration.

### 2.1.3 Use of EMS 5000 Oscillating Tissue Slicer for sections of agarose filled pig and mouse lung tissue

The oscillating tissue slicer (figure 2.5) can be used for sectioning slices from fresh or fixed tissue for use in electrophysiology, histology, immunocytochemistry, immunohistochemistry and imaging. The slicer can also be attached to a separate refrigeration system for fresh tissue that may require bathing in a buffer at a specific temperature. For future studies described in this thesis the tissue slicer was used to cut mouse and pig lung tissue to a 130 - 170  $\mu\text{M}$  thickness for airway contraction experiments and 75  $\mu\text{M}$  thickness (where possible) for haematoxylin and eosin staining. Slices of such thickness can be perfused easily with drugs to study airway contraction whilst maintaining intact lung tissue structures with visible airways that possess beating cilia.



**Figure 2.5. The EMS 5000 Oscillating tissue slicer with accompanying refrigeration bath.**

**Method:**

1. Segments of mouse/ pig lungs filled with agarose were cut into  $\sim 1 \text{ cm}^3$  cubes using a scalpel. Only tissue segments with airways free of agarose were cut.
2. The tissue segments were glued in position on the slicing stage using specimen adhesive glue, the tissue was positioned to be cut horizontally to show circular airways.
3. The slicing stage was submerged in the oscillating slicer tray which was filled with cold HBSS. The thickness was set to  $130 \mu\text{m}$  for mouse and  $140 - 170 \mu\text{m}$  for pig depending on tearing risk of the slices and staged tissue segment. The first few slices were discarded until flat, even slices were obtained. The slices were stored in cold HBSS buffer in a six well plate for 30 minutes, to be later transferred to DMEM for incubation.
4. Once all slices were cut, they were transferred to a 24 well culture plate, submerged in DMEM (Gibco Inventory no. 5769) supplemented with 2 mM glutamine, Penstrep (2.4 ml/l, Penicillin 10.000 U/ml + Streptomycin 10.000  $\mu\text{g/ml}$ ) and Fungizone (4.8 ml/l, Amphotericin B 250 UG/ml) and incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . After cutting, slices were left to settle overnight prior to experiments the following day. Airway contraction experiments were performed one - three days after preparation. However, slices were deemed viable (based on appropriate behavioural response to agonist) for up to six days in cultured conditions (data not shown (Bergner and Sanderson 2002)).

#### **2.1.4 Haematoxylin & Eosin (H&E) staining of lung slice**

Haematoxylin stains basophilic structures blue-purple, while eosin stains eosinophilic structures bright pink. Staining was applied to the control and sensitised mouse lung slices to observe structural alterations in epithelial and smooth muscle layers and view inflammatory infiltration.

##### **Materials:**

- Haematoxylin (cat. no. 51275, 100 ml, Sigma-Aldrich, Chemie GmbH), Eosin (cat. no. A5025, Batch DO2. T1105, NUSTAIN Dept. Pathology, University Hosp. Nottingham). Paraformaldehyde (cat. no. P6148, Sigma-Aldrich). Distilled water.

##### **Method:**

1. Lung slices were cut 20  $\mu$ m thick using a cryostat tissue slicer.
2. The slices were submerged in paraformaldehyde (three drops) in a petri dish for 3 minutes.
3. The slices were then transferred to a microscope slide, six drops of Haematoxylin was pipetted on the slice until completely covered, it was then washed by submerging the slide in distilled water.
4. The slice was then positioned on another dry microscope slide and two drops of Eosin were pipetted on the slice until completely covered, the slice was then submerged in distilled water to wash.

5. Images of the slices were taken using a Nikon DIAPHOT 300 microscope at 100 and 200x magnification.

### **2.1.5 Measurement of contractile response in airways**

To ensure a standard of quality control for airway contraction experiments, intact airways with beating cilia and a lumen free from agarose were chosen. For Chapter Three, airways were tested for contraction prior to experiments, a contractile response of  $> 25\%$  (in comparison to initial lumen area) to  $10\ \mu\text{M}$  ACh was deemed suitable for experiments. Chapter Four included all results for contraction measurements in sensitised mouse airways and after cytokine incubation, with the parameter that cilia were beating and airways were free of agarose.

#### **2.1.5.i Chemicals/materials required for all mouse and pig lung slice contractile experiments:**

- Microscope slides (Fisher Brand ground edges, 76 mm x 26 mm, 0.8 mm - 1.0 mm thick FB59620, UK). Cover slips (200 pcs cover glass 22 x 22 mm cat. no. 631-0124 thickness no. 1 Borosilicate glass, VWR International). Grease (Dow Corning high vacuum grease cat. no. 2273554-1 ea 5.3 oz tube, Sigma-Aldrich, Germany).
- *2 mM Calcium buffer*: HEPES minimum 99.5% titration 10 mM (H3375-5009, Sigma, Germany), magnesium chloride 1 mM (M2670-5009, Sigma, Germany), calcium chloride 2 mM (Code c/1500, FSA Laboratory Supplies,

Loughborough, UK). Hank's Balanced Salt Solution (HBSS) ((without  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , Ph red (10x) (CE) 500 ml cat. no. 14185, Invitrogen Ltd, UK), pH to 7.4 with NaOH.

- *0 mM Calcium buffer*: HEPES minimum 99.5% titration 10 mM (H3375-5009, Sigma, Germany), magnesium chloride 1 mM (M2670-5009, Sigma, Germany), HBSS (without  $\text{Ca}^{2+}$   $\text{Mg}^{2+}$ , Ph red (10x) (CE) 500 ml cat. no. 14185, Invitrogen Ltd, UK), EGTA 1 mM (E4378, Sigma, Germany), pH to 7.4 with NaOH.
- *DMSO Dimethylsulfoxide*  $\geq$  99.9% (cas. 67-68-5, Sigma, Germany). *Acetylcholine chloride* (made up in  $\text{H}_2\text{O}$ , A-6625 25 g, Sigma, Germany). *Methacholine chloride* (made up in  $\text{H}_2\text{O}$ , cat. no. 190231 MD Biomedicals, USA). *Serotonin hydrochloride* (made up in  $\text{H}_2\text{O}$ , H-9523–100 mg cas. 153-98-0, Sigma, Germany). *Histamine dihydrochloride* (made up in  $\text{H}_2\text{O}$ , H7250-5 g cas. 56-92-8 Sigma, Germany). *Caffeine* (made up in HBSS directly, sonicate well, c0750-100 g, cas. 58-08-2 Sigma, Germany). *Nifedipine* (made up in  $\text{H}_2\text{O}$ , kept on ice, N-7634-1g, Sigma, Germany). *Nickel chloride* (made up in  $\text{H}_2\text{O}$ , light sensitive, N-5756-100g, Sigma, Germany). *2-Aminoethoxydiphenylborate (2-APB)* (made up in DMSO, light sensitive, 100 mg cas. 524095-8, cat. no. 100065, Calbiochem). *Thapsigargin* (made up in DMSO, light sensitive, T9033-1MG, Sigma, Germany), *Cyclopiazonic acid* (made up in DMSO, C1530, Sigma, Germany).



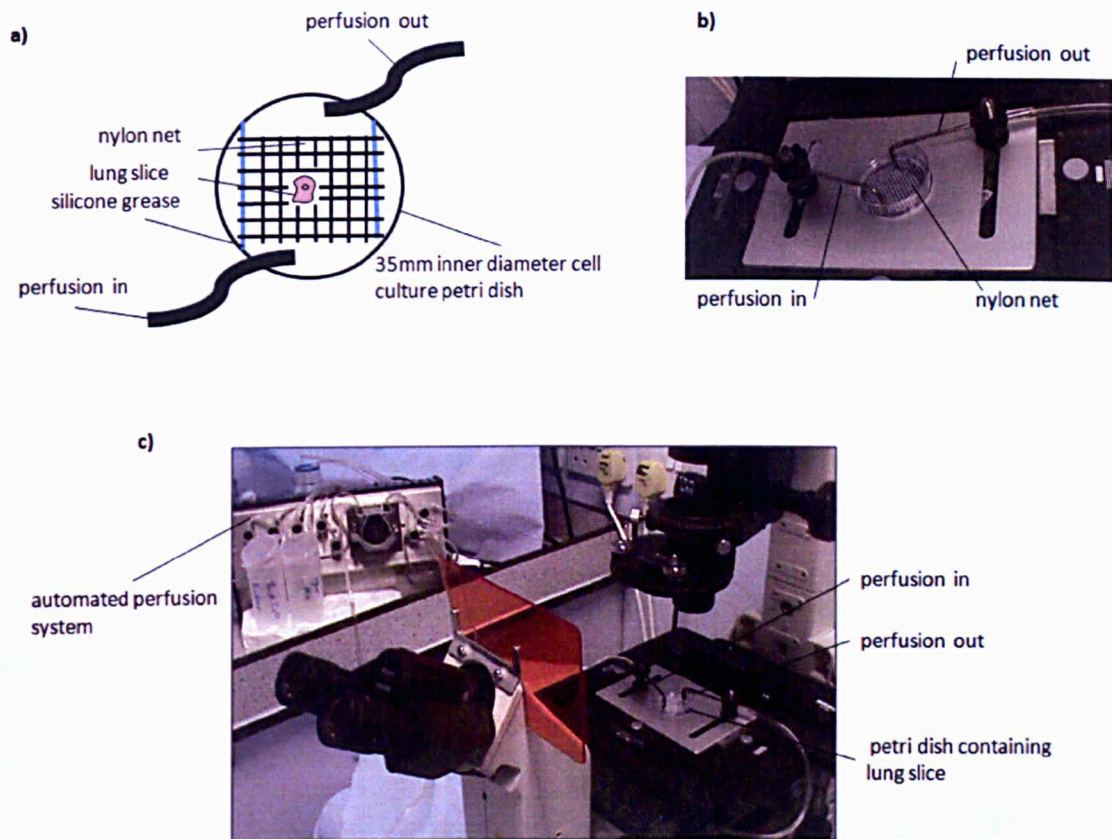
**2.1.5.ii Set up of perfusion system for contractile response experiments**

A Scientifica Perfusion Control System (Version 1.10.16) was used to perfuse varying concentrations of drug through the slices. The system can be set up for eight different samples and can be controlled manually or programmed with protocol and pump speed using the perfusion LINLAB software for windows XP.

**Method:**

1. The perfusion system was set up with chosen chemicals for each experiment (specific protocols are included with each chapter), all perfusion system tubing ranged from 0.5 - 1 mm in diameter. Universal tubes containing buffer and 1 or 10  $\mu\text{M}$  acetylcholine/methacholine, used to induce maximum contractile response were also attached to the perfusion system.
2. The slices were prepared for the perfusion system by using a 35mm diameter petri dish and creating two parallel lines in the dish with silicone grease (figure 2.6). A small amount of HBSS buffer was pipetted into the centre of the petri dish and the slice (pig/mouse) was floated out into this buffer, the airways were located and nylon mesh was placed over the slice allowing a hole over the airways. A cover slip was placed over the slice and mesh, to ensure that it overlapped the grease and prevented any leaks from buffer. The petri dish was placed in position on the microscope stage.
3. The perfusion system was connected to the stage via in and outlet tubing connections. The perfusion system was set to a pump speed of 2 ml/minute prior to recording.





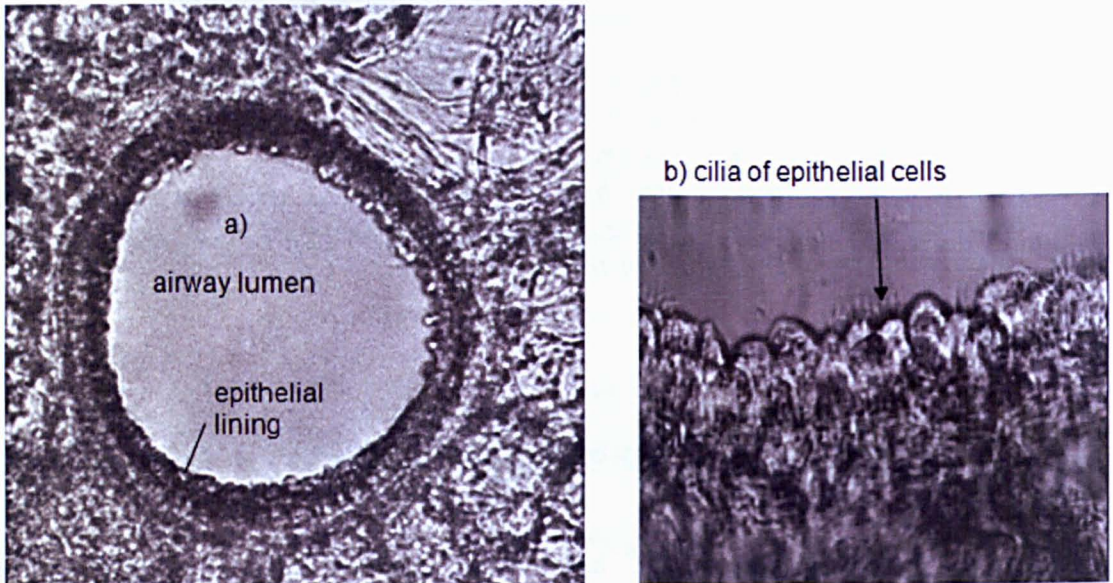
**Figure 2.6** Set up for lung slice to be attached to the perfusion system. a) The lung slice was floated in HBSS buffer and held in place under nylon mesh on a petri dish. A coverslip was placed over the top to create an airtight seal, allowing for drug perfusion across the lung slice. b),c) The petri dish was held in place on a stage over an inverted microscope, with tubing attachments for a perfusion system to allow flow directly into the petri dish, over the lung slice.

### 2.1.5.iii Measuring the airway contractile response

To capture images of airway contractions, a laser scanning confocal microscope (Zeiss Axio Observer D1, Hamatsu electron multiplier CCD camera C19100-13), with a Yokogawa Spinning disk system was used. However, it was only used on bright field settings.

**Method:**

1. Once the airway of interest was located and in focus under the microscope, the imaging software 'Volocity' was opened and set to DIC transmitted light and caption at 0.5 or 1 time point per second (specified) (figure 2.7).
2. The perfusion system (PPS perfusion system, Scientifica, LINLAB software) which was attached to the microscope stage was set to a pump speed of 2 ml/minute and using manual control, buffer was perfused through the slices.
3. Using Volocity software, airway recording began with buffer being perfused through the slices (to set initial baseline relaxed airway measurement). The chemical protocol was then followed after 1 minute of buffer perfusion.



**Figure 2.7 Microscope images of mouse airways showing airway lumen and epithelial cells.** a) image taken using bright field, 100x magnification, airway lumen diameter  $\sim 100 \mu\text{m}$ . b) During live imaging beating cilia are visible by eye on the epithelial lining, indicating healthy airways, 630x magnification (image taken by Michael Sanderson, University of Massachusetts, Worcester, US).

## **2.1.6 Analysis of contractile responses**

### **2.1.6.i Measurement of airway lumen area using Volocity analysis software**

The Volocity analysis software (PerkinElmer, USA) was used to measure airway lumen area. Volocity can be used for 2D, 3D and 4D imaging to identify, measure and track biological systems and will accept images from confocal and wide field microscopes. Using the measurements tool on the airway video clips, different methods of analysis were chosen depending on the shape and light intensity of the airway image. The software produces an area value ( $\mu\text{m}^2$ ) for every time point (one or 0.5 images/second, as stated) taken presented in the form of a spread sheet which can be exported into Excel software for further analysis.

#### **Method:**

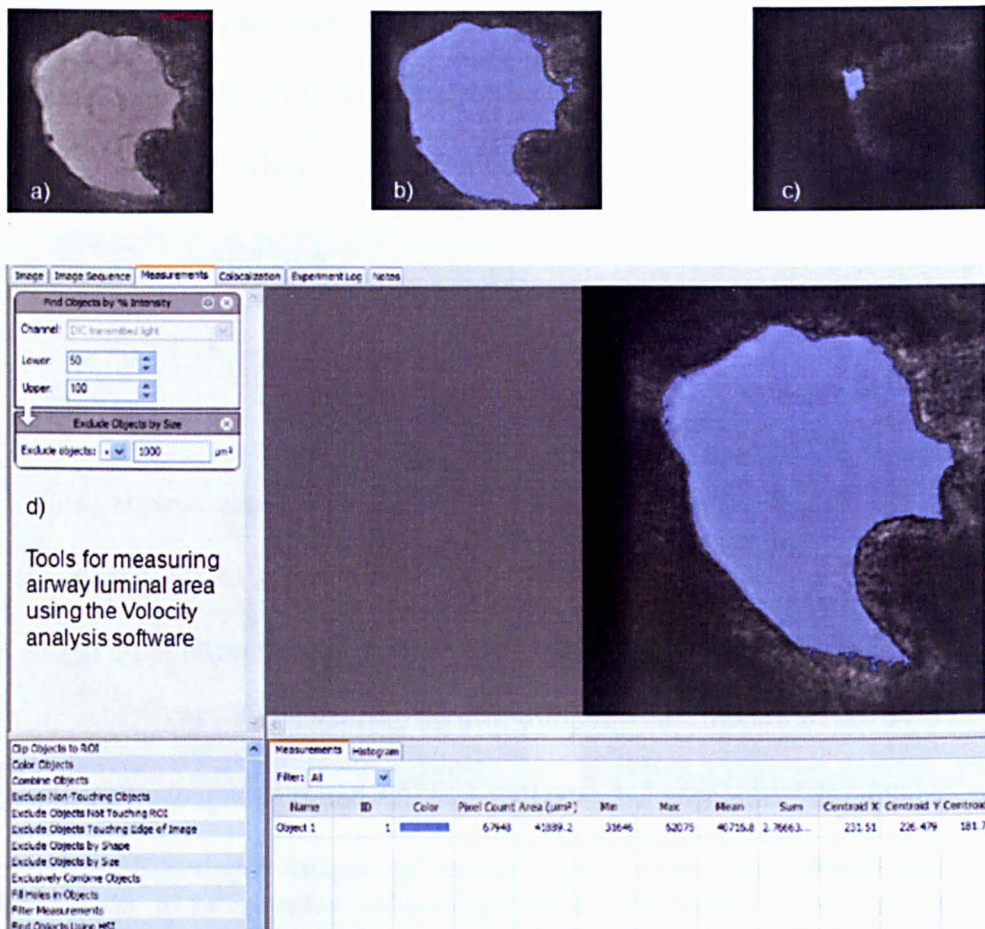
1. With the video clip of the slice opened, the measurements tool was opened. Airway luminal area was measured initially using the tool for measuring % of light intensity and contrast between the lumen and surrounding tissue, adjusted to the appropriate balance required for each slice. Other objects less than or greater than a specified size were excluded. Holes in the airway were filled using the 'fill holes tool'.
2. Measurement of all time points gave a list of values for all time points that includes airway luminal area, pixel count of airway lumen measured, perimeter and various other parameters. From these lists a method to analyse and discard incorrectly measured segments can be chosen. Most of these slices were further



analysed using the 'centroid' and 'area' tools and deleting values for objects measured other than the lumen (figure 2.8).

3. Once all values other than those measuring the lumen were discarded, the spread sheet of values was exported into excel for further analysis.

NB: If other images were incorrectly measured at the specified time points required, the airways were measured by hand using the region of interest (ROI) free hand tool and drawing around the airway lumen.



**Figure 2.8.** Using Volocity analysis software to measure airway luminal area. a) The initial pig airway image prior to analysis. b) Measurement of the luminal area after being perfused with buffer at the beginning of the protocol. c) Measurement of luminal area after the airway was perfused with 10  $\mu$ M ACh (for five minutes). d) The airway is shown being measured at the end of the experiment after buffer wash out, to the left are options selected to measure the airway image i.e measurement of the targeted object by grey contrast intensity and exclusion of unwanted objects by size.

### **2.1.6.ii Converting airway lumen area values to a percentage relative to maximum contractile response for Chapter Three**

Values measuring the airway luminal area were obtained using the Volocity measuring analysis software. These raw values for area were then manipulated in Microsoft Excel to show contractile responses relative to either 1  $\mu\text{M}$  ACh (submaximal concentration, included for testing of store operated calcium channel inhibitors) or 10  $\mu\text{M}$  ACh representing the maximum contractile response (agonist and concentration are stated with results). The overall maximum lumen area represented 0% contractile response (to prevent negative values) and the minimum area represented 100%: i) raw values for airway luminal area, taken from Volocity were filtered to obtain the absolute maximum and minimum area values. These values were then used in the equation:  $(100 - (((\text{raw area} - \text{min. area}) / (\text{max.} - \text{min. area})) * 100))$  to give % values of maximum contraction. ii) From the new set of values generated, data corresponding to the real time values (measured in seconds) was measured for ten time points before the addition of the next dose concentration and tabulated. For example, the ten values for buffer were taken between 50 and 59 seconds (real time at one time point/second), before the next drug was perfused through the system at 60 seconds. iii) The mean of the ten values for each dose concentration were then transported to the graphing software Prism.

### **2.1.6.iii Statistics**

The GraphPad Prism (version 5.02) program was used for analysis of all data, with tools for biostatistics, curve fitting and graphing.

In Chapters Three and Four dose response data were analysed with PRISM software Graphpad 5.02 for  $EC_{50}$  and  $IC_{50}$  using dose response vs. normalised response for variable slope. The  $EC_{50}$  (referring to agonist) or  $IC_{50}$  (referring to inhibitor) is defined as the concentration of drug that provokes a response halfway between the baseline and maximum response.

In Chapter Three all statistical significance was assessed using One-way ANOVA which compares the means of three or more unmatched groups. ANOVA measures the likelihood that the difference in means may be caused by chance. Dunnett's post test was chosen to individually compare all treatment conditions to the control data (either buffer or  $1/10 \mu\text{M}$  ACh values). The experiments were designed specifically to assess the influence of the chosen drugs within each experiment compared to the reference drug.

In Chapter Four all statistical significance was assessed using the Mann Whitney test (unless otherwise stated) for non-parametric distribution, which ranked the data and compared medians. Due to having too few data points, this was deemed the most conservative method to assess differences in airway contraction in the two lung slice groups (control vs. treatment by ovalbumin sensitisation or cytokine exposure). With such variation in results between individual mice, particularly in immune responses in the transgenic models, it was necessary to rank the results and compare between medians for significance (rather than inadvertently increasing the significance of the treatments effects by including high outliers that would increase a mean).

## 2.2 CELL CULTURE

All cell culture reagents were obtained from SIGMA (Dorset, UK), unless otherwise stated, all cell culture plastics were obtained from Corning life sciences from Fisher Scientifica (Loughborough, UK).

### 2.2.1.i Isolation of mouse trachea smooth muscle cells, collagenase method

Tracheas were used for smooth muscle cell culture from BALB/c ovalbumin sensitised mice and PBS treated controls and C57BL/6 WT naïve mice; cultures were later used to measure differences in calcium signalling between treatment groups after ovalbumin sensitisation or cytokine exposure. The following methods for isolation of the trachea were taken from previous published methods (Helli, Pertens et al. 2005) and refined by trial and error to optimise timings.

#### Materials:

- Papain (1.5 mg, cat. no. P4762, 100 mg, Sigma, Germany). DTT Collagenase (type H) (1.6 mg cat. no. c8051, 100 mg, Sigma, Germany).
- HEPES solution: per 1L of water, NaCl 120 mM, KCl 6 mM, Glucose 6 mM, Hepes 5 mM, MgSO<sub>4</sub> 0.28 mM, CaCl<sub>2</sub> 0.125 mM (all chemicals supplied by Sigma, Germany).
- DMEM (D5796, Sigma-Aldrich, Inc., St Louis, Mo., USA) supplemented with foetal calf serum (FCS) (10% (v/v)), Penstrep (2.4 ml/l, Penicillin



10.000 U/ml + Streptomycin 10.000 µg/ml) and Fungizone (4.8 ml/l, Amphotericin B 250 UG/ml). Distilled H<sub>2</sub>O.

**Method:**

1. The tracheas were collected and placed in HBSS on ice. Using a dissection microscope, the tracheas were cleaned free of connective tissue.
2. The tracheas were individually added into eppendorfs containing 1.5 mg of papain in 1 ml of HEPES solution (prewarmed to 37°C for 30 minutes).
3. Eppendorfs were centrifuged for 2 minutes at 200 rpm, papain was pipetted off.
4. Collagenase (1ml) was added (1.6 mg collagenase to 1 ml of HEPES solution, prewarmed to 37 °C for 30 minutes). Eppendorfs were again centrifuged for two minutes at 700 rpm, collagenase was pipetted off.
5. The digested tracheas were washed with HBSS 5 times by adding 1 ml of HBSS, centrifuging for five minutes at 700 rpm, taking off the HBSS and repeating with fresh HBSS.
6. Cells were pipetted up and down gently to loosen the pellet and viewed under a microscope to check for elongated airway smooth muscle cells.
7. Cells were again centrifuged and HBSS was pipetted off, 1 ml of DMEM was added (supplemented with 2 mM glutamine, FCS and Penstrep and Fungizone). Cells were then transferred to 25 cm<sup>2</sup> cell culture flasks with a total volume of 7 mls and incubated at 37°C in 5% CO<sub>2</sub>.
8. Once the cells reached confluent patches, they were dislodged from the flask surface by timed trypsinisation (leaving epithelial cells still on the flask) and

replated in a new 25 cm<sup>2</sup> surface area flask to spread evenly with ASM cells (this stage is usually reached after six weeks).

### **2.2.1.ii Isolation of mouse trachea smooth muscle cells, explants method**

Tracheas were used from WT BALB/c and C57BL/6 WT naïve mice. Although the previous method for isolation of mouse trachea smooth muscle was successful in growth of smooth muscle cells, the process took six weeks for a 25 cm<sup>2</sup> flask to reach confluency with a population of 50% epithelial cells surviving indefinitely despite the media being ASM specific. Therefore an explant method was trialled based on methods from previous publications (Hall, Widdop et al. 1992). The explant method produced a faster growing population (< three weeks) of predominantly smooth muscle (> 70%) initially. However the epithelial cells still survived the duration of the culturing. This problem was reduced by timed trypsinising. In preparation for replating, the smooth muscle was detached from the flask surface after three minutes of incubation with porcine trypsin (2 mg/ml)/EDTA (5 mg/ml) (using 3 ml/25 cm<sup>2</sup> flask), as compared to the epithelial cells which took five minutes to detach completely. This allowed the separation and collection of smooth muscle and epithelial cell populations separately.

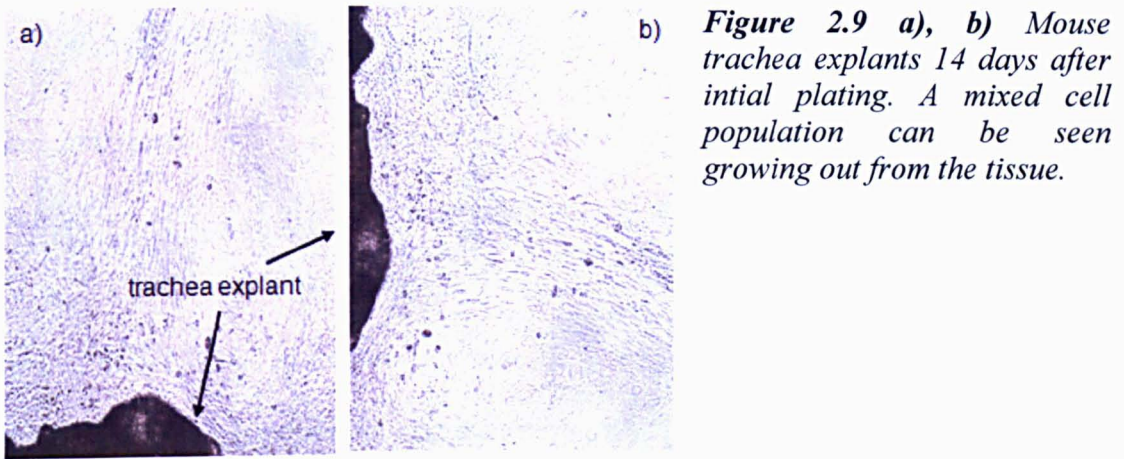
### **Materials:**

- DMEM (D5796, Sigma-Aldrich, Inc., St Louis, Mo., USA) supplemented with foetal calf serum (FCS) (10% (v/v)), Penstrep (2.4 ml/l, Penicillin

10.000U/ml + Streptomycin 10.000 µg/ml) and Fungizone (4.8 ml/l, Amphotericin B 250 UG/ml).

**Method:**

1. Similar to the approach to pig explants (section 2.2.2), all the mouse tracheas were dissected at the animal house and brought back to the lab on ice, in HBSS buffer (with 2 mM  $\text{Ca}^{2+}$ ).
2. Under the dissection microscope the tracheas were individually cleaned of connective tissue and the oesophagus, in a petri dish with a small amount of HBSS.
3. The tracheas were then transported to a sterile hood and transferred to a pot of DMEM supplemented with FCS (10% (v/v)), Penstrep (2.4 ml/l, Penicillin 10.000 U/ml + Streptomycin 10.000 µg/ml) and Fungizone (4.8 ml/l, Amphotericin B 250 UG/ml) to be cleaned.
4. Tracheas were individually taken from the pot and smeared across the petri dish, chopped in half using a scalpel, with the inner side exposed to the surface of the dish. After waiting five minutes for the trachea segments to stick down to the dish, DMEM was slowly pipetted into the dish, enough to cover the trachea segment but not to detach it from the base of the plate. Plates were incubated at 37°C in 5%  $\text{CO}_2$ .
5. The petri dishes were left undisturbed for five days, media was then changed. After five days ASM cells could be seen growing out from all tissue segments.
6. Media was changed every five days, cells were allowed to grow until confluent for RNA extraction or further passaging.



### 2.2.2 Isolation of pig trachea smooth muscle for cells, explant method

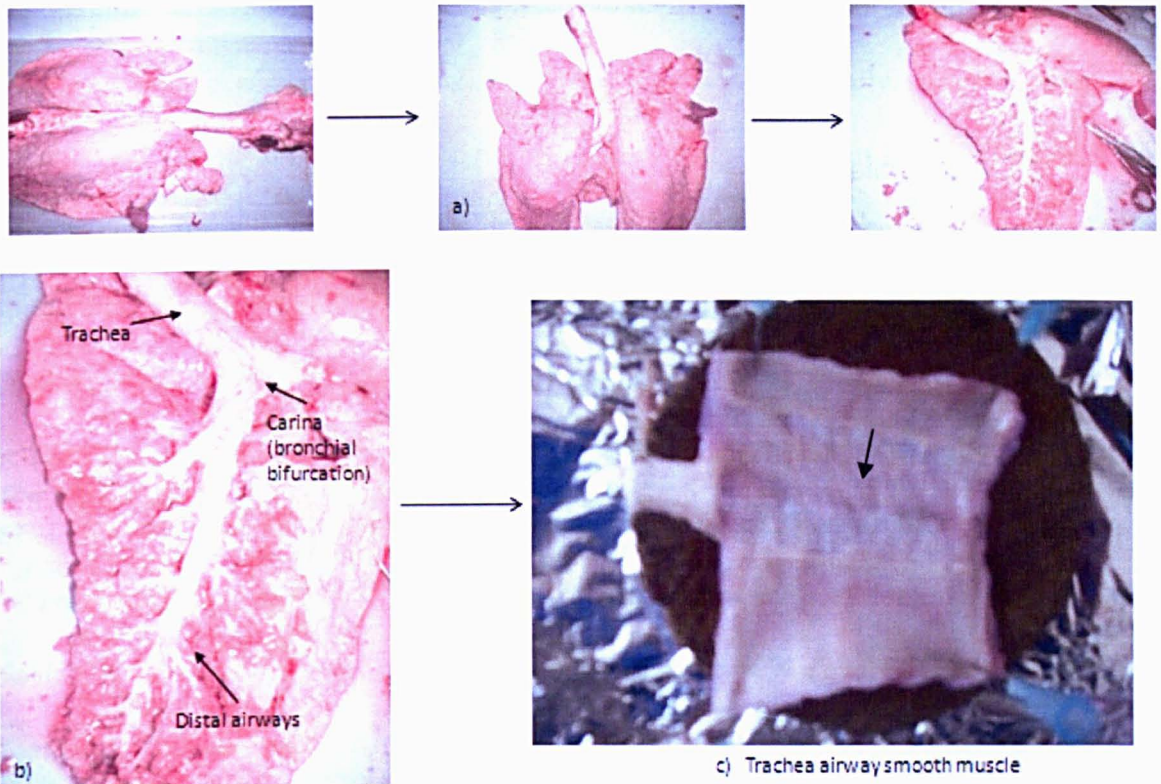
This experiment was carried out in pig as a practice for future isolation of ASM from various generations of human airways to be cultured for later calcium signalling experiments comparing the response to selected agonists and inhibitors with decreasing airway size.

#### Materials:

- DMEM (D5796, Sigma-Aldrich, Inc., St Louis, Mo., USA) supplemented with foetal calf serum (FCS) (10% (v/v)), Penstrep (2.4 ml/l, Penicillin 10.000 U/ml + Streptomycin 10.000 µg/ml) and Fungizone (4.8 ml/l, Amphotericin B 250 UG/ml).

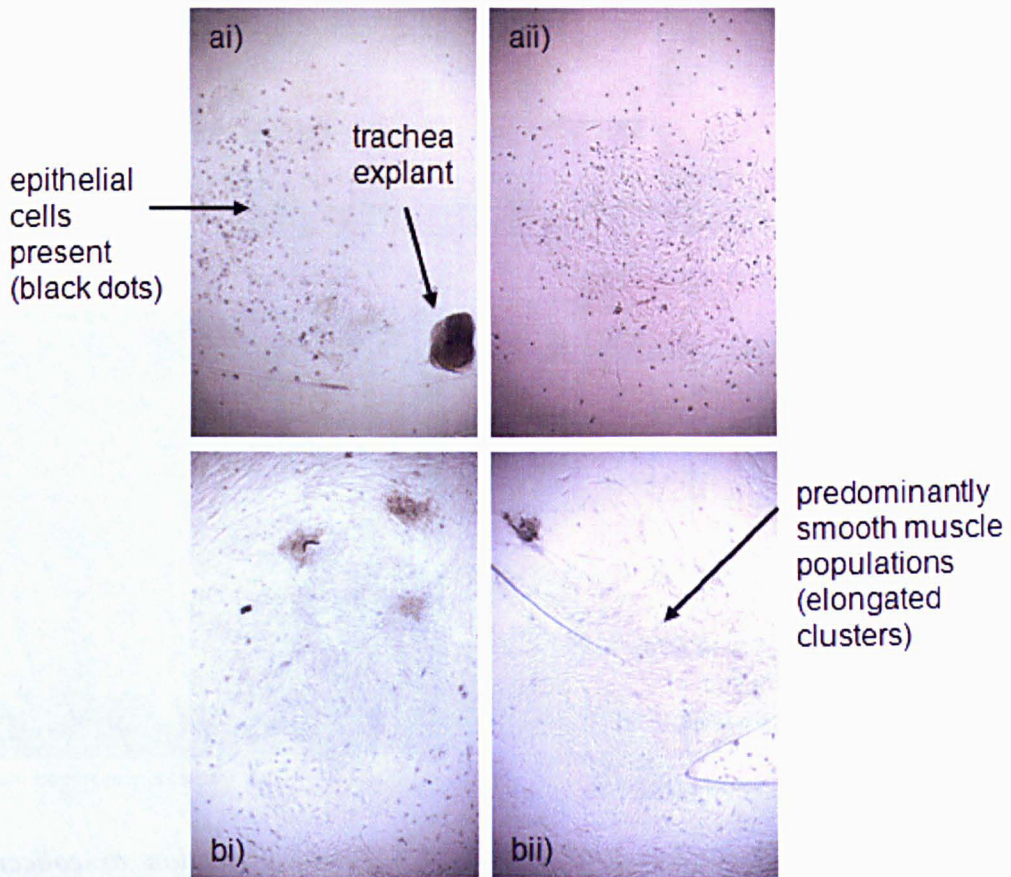
**Method:**

1. Trachea and further airways were dissected free of excess connective and lung tissue being careful not to cut the airways, the right inferior lobe was chosen for lower generation explants.
2. A segment of trachea (15 cm long) was cut, washed in DMEM with 2x Penstrep and Fungizone (PSF), then transferred to the sterile hood for further steps. The trachea segment was then cut in half and pinned down on a cork board.
3. A cut was made down either side of the striated smooth muscle segment, the covering membrane was stripped back to reveal pale pink/transparent ASM.
4. The ASM was picked off using curved scissors, four very small pieces were scraped along/dropped into each well of a six well plate until they appeared to adhere and dry on the surface (left only for five minutes). DMEM (3 ml) with FCS and supplemented 1x PSF was then pipetted very slowly into each well being mindful not to detach the ASM bundles from the bottom surface of the well. Plates were very carefully moved to the incubator and grown at 37°C in 5% CO<sub>2</sub>.
5. The bronchial and distal segments were then cut and dissected as above in the sterile hood, by stripping back the covering membrane and picking off the ASM.
6. The plates were left undisturbed for five days at which point the media was changed and loose tissue segments aspirated.
7. Media was changed every five days and cells allowed to grow until confluent.



**Figure 2.10. Pig trachea and bronchial segment dissection to collect airway smooth muscle cells.** a) Dissected adult domestic pig lungs and trachea showing seven lung lobes. b) Dissection of right inferior lung lobe to show branching airway generations: trachea, bronchial segments, distal airways. c) Segment of trachea (15 cm length), cut in half and stripped of membrane on inside of trachea to expose pale pink airway smooth muscle bundles (arrow), used for explant method.





**Figure 2.11.** *Cultured airway smooth muscle cells dissected from pig trachea and bronchial segments and grown using the explant method. Images of pig trachea ASM taken using a Diaphot 300 microscope at 200x magnification for panel ai), bi) taken a week after the tissue segments were plated. The tissue segment was visible, epithelial cells were still present; ASM cells were beginning to grow in patches. Panel aii), bii) images taken two weeks after the tissue segments were plated, most of the epithelial cells had died off and the ASM showed confluent patches.*

### 2.2.3 Isolation of human airway smooth muscle (HASM) cells

Bronchial tissue was obtained from patients with no history of asthma undergoing surgery for lung cancer, with patient and ethical approval from the Nottingham local ethical research committee. All donors used were male and ranged in age from 55 - 70. The procedure for isolating the airway smooth muscle was performed by previous members of the department, primary airway smooth muscle



populations were expanded through culture using previous published methods (Daykin, Widdop et al. 1993). Bronchial segments were surgically removed from patients and placed in DMEM (without calcium or magnesium) on ice. The smooth muscle cells were accessed by removing the surrounding connective tissue and epithelial tissue. The smooth muscle was finely chopped with a scalpel and incubated with collagenase (2 mg/ml) for two - three hours at 37°C. Every hour the cells were filtered through a 100 µm cell strainer, a pellet was collected by centrifuging, cells were transferred to a 25 cm<sup>2</sup> culture flask. For the first two weeks the cells were maintained in DMEM (with 4500 mg glucose/ L pyridoxine, HCl and NaHCO<sub>3</sub>, SIGMA cat. no. D5671) supplemented with 10% (v/v) FCS and 2 mM Glutamine, penicillin (200 U/ml), streptomycin (200 µg/L) and amphotericin B (0.5 µg/L) at 37°C, 5% CO<sub>2</sub>. After two weeks penicillin, streptomycin and amphotericin were removed from the culture media and cells were left to reach confluency. Cells were stored in liquid nitrogen in FCS with 10% (v/v) DMSO until required for experiments.

When taken out of liquid nitrogen the cells were thawed and centrifuged at 1200 xg for five minutes, using an MSE Mistral 3000i centrifuge (Sanyo Gallenkamp, Loughborough, UK) to remove FCS and DMSO, the cells were plated in DMEM in 75 cm<sup>2</sup> culture flasks. Media was changed every three days, when confluent the cells were passaged by removing the media, using 3 ml of prewarmed porcine trypsin (2 mg/ml)/EDTA (5 mg/ml) to detach the cells from the flask. Cells were transferred to a new universal tube in DMEM and centrifuged at 1000 rpm for five minutes, the trypsin and media was removed. The cells were then resuspended in media and replated in a new 75 cm<sup>2</sup> culture flask. Cells were routinely split 1:4

and allowed to grow to confluence, then passaged further. Cells between passage two and seven were used for experiments.

#### **2.2.4 Culture of human bronchial epithelial cells (HBEC)**

HBECs were purchased from Bio Whittaker (Cambrex Bioscience, MD, USA), taken from donors with no history of lung disease. Cells were cultured in bronchial epithelial cell basal medium (BEBM) (Cambrex Bioscience) supplemented with human recombinant EGF (0.5 ng/ml), epinephrine (0.5 ng/ml), retinoic acid (0.1 ng/ml), triiodothyronine (6.5 ng/ml), amphotericin (50 ng/ml), bovine pituitary extract (52 µg/ml), hydrocortisone (0.5 µg/ml), transferrin (10 µg/ml), insulin (5.0 µg/ml) and gentamicin (50 µg/ml). HBECs were maintained at 37°C and 5% CO<sub>2</sub>, cells were passaged within 24 hours of being confluent. Soybean trypsin inhibitor (0.5 mg/ml) was used to neutralise trypsin when detaching the cells. Cells were cultured to passage three and four for experiments.

## **2.3 MOLECULAR BIOLOGY**

### **2.3.1 RNA extraction from mouse lung slices (PBS and ovalbumin sensitised) using Nucleospin RNA XS kit**

RNA was extracted from mouse lung slices from PBS matched and ovalbumin sensitised mice after acute (PBS = four mice, OVA = five mice), mid-chronic (PBS = eight mice, OVA = eight mice) and chronic protocols (PBS = four mice, OVA = four mice). RNA extraction from tissue was initially trialled using an alternative kit (Qiagen RNeasy mini protocol and Qia-shredder columns), the tissue was also passed up and down through a 2 mm needle; these experiments did not successfully separate the tissue. After optimisation of timings, a more successful approach involved the slices (five slices at 130  $\mu\text{m}$  thickness each, per mouse, per eppendorf) being initially incubated in collagenase H, to allow break down of structures (also using a spatula (0.25 cm diameter)). Total RNA was then isolated and purified from the mouse lung slices using the Nucleospin RNA XS kit (Macherey-Nagel) following the manufacturers' instructions. The Nucleospin XS kit is designed to isolate total RNA from very small samples including micro dissected tissue or small amounts of cells. It uses a column design incorporating a funnel shaped ring that holds a smaller silica membrane which allows for smaller elution volumes.

**Materials:**

- Nucleospin RNA XS kit (DNase included) (cat. no. NZ74090250, Fisher, UK). Collagenase H (cat. no. C801, Sigma, Germany). Ethanol (96-100% (v/v), (Medical School stores cat. no. LH-M0120E).
- Hank's Balanced Salt Solution (HBSS) (without  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , phenol red, cat. no. 14175-095, Gibco).

**Method:**

- 1- For every individual mouse lung set five lung slices (130  $\mu\text{m}$  thick, equivalent of 1 - 5 mg of tissue) were transferred into 500  $\mu\text{l}$  of collagenase H (1.5 mg in HBSS) in a 1.5 ml eppendorf tube and incubated at 37°C for five - ten minutes or until all the tissue had broken into smaller pieces (occasionally slices were pipetted up and down to break tissue). They were centrifuged at 100 rpm for seven minutes and collagenase H was pipetted off.
- 2- Steps were then followed according to the Nucleospin XS protocol. 200  $\mu\text{l}$  of Buffer RA1 and 4  $\mu\text{l}$  TCEP was added to the tissue sample and pipetted to mix.
- 3- Carrier RNA working solution (5  $\mu\text{l}$ , 20 ng) was added to the lysate and mixed by vortexing, then centrifuged.
- 4- The Nucleospin filter column was placed in a 2 ml collection tube, the lysate was applied to the column and centrifuged for 30 seconds at 11,000 rpm.
- 5- The Nucleospin filter column was discarded and 200  $\mu\text{l}$  of 70% (v/v) ethanol was added to the homogenized lysate and mixed by pipetting up and down.

- 6- A nucleospin RNA XS column was placed into a 2 ml collection tube and the lysate was loaded, it was centrifuged for 30 seconds at 11,000 rpm. The lysate was discarded and transferred to a new collection tube.
- 7- Membrane desalting buffer (100  $\mu$ l) was added and centrifuged at 11,000 rpm for 30 seconds to dry the membrane.
- 8- Reconstituted DNase (3  $\mu$ l) was added to a 27  $\mu$ l reaction buffer for rDNase for each sample. They were mixed by gently flicking the bottom of the tube.
- 9- DNase reaction (25  $\mu$ l) mixture was applied directly onto the centre of the silica membrane of the column and incubated at room temperature for 15 minutes.
- 10- Buffer RA2 (100  $\mu$ l) was added to the Nucleospin RNA XS column. This was incubated for two minutes at room temperature then centrifuged for 30 seconds at 11,000 rpm. The column was placed into a new collection tube.
- 11- Buffer RA3 (400  $\mu$ l) was added to the column, then centrifuged for 30 seconds at 11,000 rpm.
- 12- Buffer RA3 (200  $\mu$ l) was added to the column then centrifuged for two minutes at 11,000 rpm to dry the membrane. The column was then placed into a nuclease free 1.5 ml tube.
- 13- The RNA was eluted in 10  $\mu$ l of RNase free H<sub>2</sub>O by centrifuging at 11,000 rpm for one minute. The RNA was stored at -80°C.

### **2.3.2 RNA isolation from cultured HASM, HBEC, mouse trachea smooth muscle**

Total RNA was isolated and purified from mouse trachea smooth muscle cells, human airway smooth muscle cells (HASM) and human bronchial epithelial

cells (HBEC) using the RNeasy mini kit and QIAshredders (Qiagen, Sussex, UK). RNeasy kits are designed to isolate total RNA from small quantities of starting material. The purified RNA can be used for RT-PCR and cDNA synthesis. With this particular procedure, all RNA molecules longer than 200 nucleotides are isolated. The manufacturer's protocol was followed (RNase free DNase Set cat. no. 79254, Qiagen, QIAshredder Homogenisers cat. no. 79654, Qiagen).

**Method:**

- 1) Unless stated otherwise, cells were harvested from a single confluent T75 flask. Once confluent, cells were washed with PBS, a buffer containing guanidine thiocyanate and 1% (v/v) Beta-mecaptoethanol 600 µl was added to detach cells from the flask surface, they were then scraped from the flask and transferred for further homogenisation by passing through a QIAshredder.
- 2) The QIAshredder spin column was placed in a 2ml collection tube and the lysate was applied (maximum 700 µl) to column. It was centrifuged for two minutes at 13,000 rpm.
- 3) 70% (v/v) ethanol (600 µl) was added to the homogenised lysate and mixed by pipetting. The lysate was kept for the next steps.
- 4) Up to 700 µl of the sample was applied to an RNase mini column placed in a 2 ml collection tube and centrifuged at maximum speed for 15 seconds. The flow through was discarded (RNA should be bound to membrane).
- 5) Buffer RW1 (350 µl) was added into the RNeasy column and centrifuged at maximum speed for 15 seconds. The flow through was discarded.

- 6) DNase I stock solution (10  $\mu$ l) was added to 70  $\mu$ l of Buffer RDD and mixed gently by inverting the tube.
- 7) This DNase I incubation mixture (80  $\mu$ l) was added directly onto the RNeasy column silica-gel membrane and left at room temperature for 15 minutes.
- 8) Buffer RW1 (350  $\mu$ l) was added into the column and centrifuged for 15 seconds at maximum speed. The flow through was discarded.
- 9) The column was transferred to a new 2 ml collection tube. 500  $\mu$ l of Buffer RPE was added onto the column, this was centrifuged at maximum speed for 15 seconds, the flow through was discarded.
- 10) Another 500  $\mu$ l of Buffer RPE was added to the RNeasy column, this was centrifuged for two minutes at maximum speed to dry the silica-gel membrane, the flow through was discarded. The sample was centrifuged again for a further one minute at maximum speed.
- 11) The column was transferred to a new 1.5 ml microfuge tube. 50  $\mu$ l of RNase-free water was added directly onto the membrane. This was centrifuged for one minute at 13,000 rpm to elute.

All RNA samples were quantified using a NanoDrop ND100 spectrometer (NanoDrop technologies, Rockland, DE, USA), which requires 1  $\mu$ l of RNA for analysis. The 260/280 ratio serves as a measure of RNA purity with respect to contamination from protein. The 260/230 ratio reflects contamination from reagents such as salts. An appropriate value for both ratios is between 1.80 - 2.1. All lung slice RNA and cell cultured RNA used for cDNA synthesis had values within the appropriate quality control parameters.



### 2.3.3 cDNA synthesis

RNA (0.6 µg) was reversed transcribed to form first strand cDNA using the Superscript First Strand Synthesis kit (Invitrogen, Cambridge Bioscience) and random hexamers following manufacturers' instructions. Reverse transcriptase negative (RT-) samples were prepared as negative controls for genomic contamination. Superscript First Strand synthesis System for RT-PCR is used to generate first strand cDNA from total or poly(A) + RNA. In conjunction with PCR the system can be used to quantitate the amount of specific mRNA from small numbers of cells or to clone specific cDNAs.

#### **Materials:**

Superscript First Strand Synthesis System of RT-PCR (cat. no. 110904-018, Invitrogen, UK). DEPC-treated water (cat. no. 10977-015, Invitrogen, UK). RNA samples.

**Method: example method for cDNA synthesis, for mouse trachea smooth muscle (TSM)**

1. The final concentration of RNA (600 ng/µl) was determined using values taken from the NanoDrop spectrometer readings. RT- samples were also generated (see below).

2. Template master mixes were made up:

<b>Mouse TSM (130 ng/μl)</b>	<b>1x (μl) RT+</b>	<b>1x (μl) RT-</b>
RNA (DNA free)	5.81	5.81
Random Hexamers (50 ng/μl)	1	
10 mM dNTP	1	
DEPC water	2.19	14.19 (to make up 20 μl total volume)
<b>Total</b>	<b>10</b>	<b>20</b>

3. Template master mixes were incubated at 65°C for five minutes.

4. The RT master mix was made up.

<b>RT Master Mix</b>	<b>1x (μl)</b>	<b>6x master mix (for RT+ only)</b>
10x RT Buffer	2	12
25 mM MgCl <sub>2</sub>	4	24
0.1 M DTT	2	12
RNase Out (40 U)	1	6
<b>Total</b>	<b>9</b>	<b>54</b>

5. New eppendorfs for each cell set were labelled, 9 μl of RT master mix and 10 μl of template master mix were added. Samples were incubated at 25°C for two minutes on a heat block.

6. Superscript II reverse transcriptase (1 μl, 50 U) was added to the RT + samples only (20 μl total).

7. Samples were incubated at 25°C for ten minutes, 42°C for 50 minutes and 70°C for 15 minutes using a PCR block, then chilled on ice for one minute.

8. Samples were centrifuged briefly and 1 μl of RNase H/tube (2 U) was added. Samples were then incubated at 37°C for 20 minutes.

9. Finally the samples were diluted in DNase free H<sub>2</sub>O, from the 20 μl reaction to 100 μl and stored at -80 or -20°C.

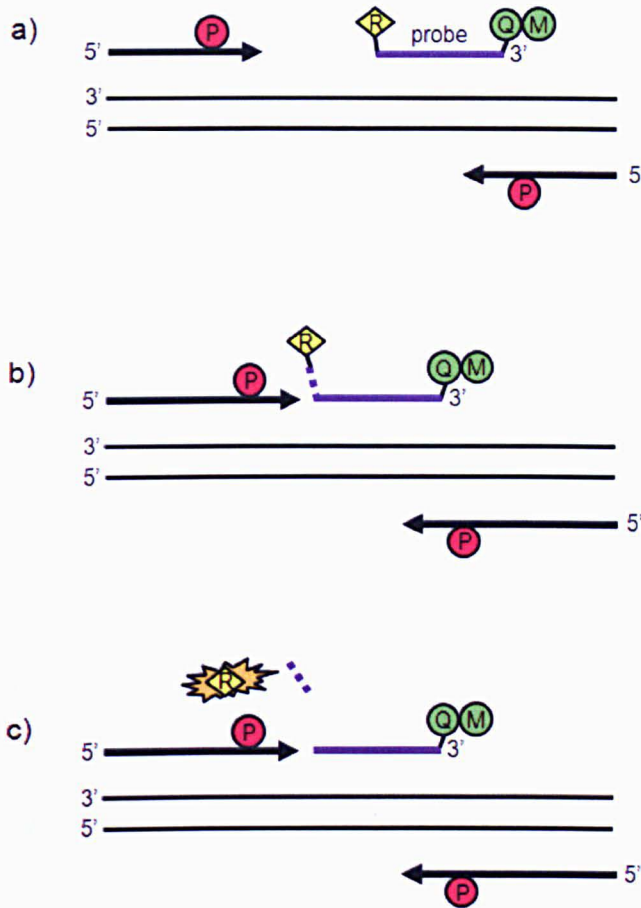
10. All samples were checked for synthesis or contamination using RT-PCR with such housekeeping primers as GAPDH,  $\beta$ -actin or 18S and by further using these products for agarose gel electrophoresis to show bands at the predicted size for RT+ samples and show RT- samples without bands present.

### **2.3.4 Quantitative Taqman® PCR (Q-PCR) in lung tissue from sensitised mice**

Methods for relative quantification of mRNA expression allow comparisons of the expression level of a specific target gene between different samples. Q-PCR measures PCR amplification as it occurs, as opposed to PCR.

The Taqman® process measures the fluorescence emitted by a probe specific for the target sequence. PCR is performed on a DNA template, which can be single or double stranded. The probe is labelled with a 5' reporter and 3' quencher dye (with minor groove binder (M), which increases the melting temperature without increasing the probe length) and is designed to anneal specifically to a complementary sequence between the forward and reverse primer sites during PCR. When the probe is intact, the close proximity of the reporter dye to the quencher dye results in suppression of the fluorescence emitted by the reporter dye. During PCR high temperature is applied to separate the DNA, the temperature is then lowered to let the primers anneal to the template, finally the temperature is increased to allow the polymerase to extend the primers (Kubista, Andrade et al. 2006; Lind, Stahlberg et al. 2006). During the extension phase the 5' nuclease activity of the Taq DNA polymerase cleaves the probe when it is hybridised to the target. Cleavage separates the reporter dye from the quencher dye, allowing increased fluorescence by the reporter (figure 2.12). The measured fluorescence serves as quantification of

expression of the target gene (Afonina, Zivarts et al. 1997; Livak and Schmittgen 2001).



*Figure 2.12 Quantitative PCR process. a) the Taqman® probe is labelled with a reporter dye (R) and a non fluorescent quencher (Q) with a minor groove binder (M). The probe is designed to anneal to the target sequence at a site between the forward and reverse primers. When the probe is intact reporter fluorescence is suppressed. b) the hot start DNA polymerase (P) cleaves the probe separating the reporter from the quencher, allowing fluorescence of the reporter. c) polymerisation of the strand continues to completion. The level of fluorescence emitted indicates the amount of target amplified.*

### Materials:

- 96 well plates, 8 well strips, caps (Stratagene 410088 & 401425 Medical School Store, University of Nottingham). Universal PCR Master mix (20x) (cat. no. 1275, Applied Biosystems). cDNA of interest. Primers and Probes (listed in tables 2.1 and 2.2). Predeveloped Assay Reagents (PDAR) (Applied Biosystems).

Assays were designed to target murine IL-33 and the corresponding receptors ST2 soluble form (ST2s) and ST2 transmembrane form (ST2L) and key interacting proteins involved in ASM stored operated calcium entry, Stromal Interaction Molecule 1 (STIM1) and Orai1. cDNA samples created used RNA isolated from lung slices obtained from mice that had undergone the acute, mid-chronic and chronic sensitisation protocol along with matched PBS controls. Samples were analysed by quantitative PCR using the Mx300SP real-time thermal cycler (Stratagene, La Jolla, California). Expression from each cDNA sample was normalised to  $\beta$ -actin expression (used as a house-keeping gene to correct for equal cDNA input) using a pre-designed assay (Applied Biosystems, Foster City, California), initially 18S was used as a second housekeeping gene to compare against  $\beta$ -actin to ensure uniform expression across all samples, both housekeeping genes gave similar results. Using Beacon designer 7 (Stratagene) and manual alignment between mRNA sequence and full length transcripts, all primer and probe assays were designed to span an intron-exon boundary (to eliminate genomic DNA contamination). Primers were purchased from Invitrogen. The probes were dual labelled with 5' FAM and 3' TAMRA dyes (unless otherwise stated) and were purchased from Applied Biosystems, also with the universal master mix and the  $\beta$ -actin and 18S pre-designed assays (PDAR). Each sample was run in triplicate using 900 nM forward and reverse primers and 250 nM probe. RT- samples and no template controls were analysed also. A standard protocol was used to detect amplification consisting of two minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. The list of primers and probes used for comparison of gene expression between PBS matched and ovalbumin sensitised acute, mid-chronic, chronic mouse lung tissue is provided (table 2.1).

**Table 2.1 Primers and probes used for quantification of mRNA between PBS matched and ovalbumin sensitised acute, mid-chronic and chronic mouse lung tissue.** NFQ: Non-fluorescent Quencher, MGB: Minor groove binder, PDAR: pre-designed assays, bp: base pairs, N A: data not available. All primers (40 nmol) were purchased from Invitrogen, all probes (40 nmol) were purchased from Applied Biosystems.

Gene	Forward primer	Reverse primer	Probe	5' label	3' label	Amplicon	Exons targeted
IL-33	CAAGA CCAGG TGCTAC TACGC	TGGGA CTCAT GTTCA CCATC AC	TCTTC CCATC CACAC CGTCG CCTGA	FA M	TA MR A	100bp	2 - 4
ST2s	TTAAG AACTG CAAAG CTCTCC AAG	ACAAG TGTA TCACC TTCAT CATC	CCAAG GTTC GGGCA CACAG GTCC	FA M	TA MR A	101bp	2 - 4 at 583 - 683bp
ST2L	CATTTA TGGGA GAGAC CTGTTA CC	GTCTT CTGCT ATTCT GGATA CTGC	CACCA CGGTG GCTGC ATCTT GCC	FA M	TA MR A	77bp	9 - 10 at 1391 - 1467bp
Orai1	TCTACT TAAGC CGCGC CAAG	ACTTC CACCA TCGCT ACCAT G	TCAAA GCTTC CAGCC GGACC TCGG	FA M	TA MR A	85bp	1 - 2 at 426 - 510bp
STIM1	TGGAC ACAGT GCTGTT TGGG	TGTGC TCCTT AGAGT AACGG TTC	CTCCT CTCTT GACTC GC	FA M	NFQ (MG B)	138bp	1175 - 1312bp
18S, $\beta$ -actin	PDAR			HEX	N A	N A	N A

**Method: example Quantitative PCR protocol, using cDNA from OVA sensitised mouse lung tissue**

For OVA sensitised samples RT + (1,2,3,4), OVA sensitised RT- and PBS RT + (1,2,3,4) cDNA samples (nine samples) from RNA extracted from mouse lung slices for quantifying expression of IL-33, normalised to  $\beta$ -actin.

## 1. cDNA master mixes were made up

OVA RT +, PBS RT +, OVA RT -	x1 (µl)	x3.5 (for triplicate for same gene set) x3 for all 3 gene sets (IL-33, 18S, β-actin)
Template cDNA	1	10.5
Template H <sub>2</sub> O	3	
H <sub>2</sub> O	1.9	51.45
Universal master mix	10	105
Total	15.9	166.95

## 2. Primer/probe master mixes were made up

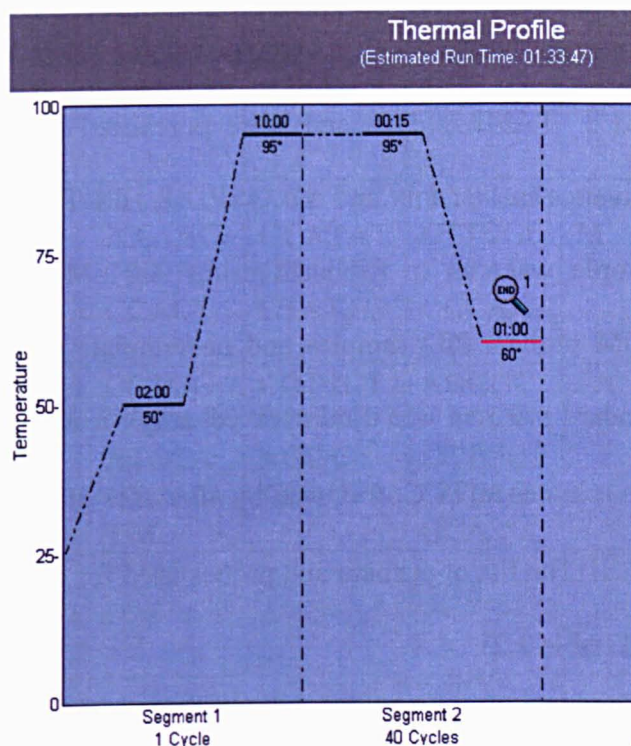
IL-33	x1 (µl)	x33	18S, β-actin	x1 (µl)	x33
Forward primer	1.8	59.4	PDAR	1	33
Reverse primer	1.8	59.4	H <sub>2</sub> O	3.1	102
Probe	0.5	16.5	Total	4.1	135.3
Total	4.1	135.3			

Reference PDAR requirements (for 20 µl reaction)
Template cDNA 4 µl
H <sub>2</sub> O 5 µl
Universal master mix 10 µl
PDAR 1 µl

- Primer master mix (4.1 µl) was added into each tube/well for the corresponding gene (IL-33, 18S or β-actin) (template controls were included). The cDNA master mix (15.9 µl) was added to the corresponding tube/well (reagent/H<sub>2</sub>O controls were included). Plates were centrifuged at 1000 rpm for five minutes.
- Using the Taqman® software MxPro- Mx300SP: The plate set up was checked, making sure HEX (corresponding to PDAR housekeeping genes) and FAM (for IL-33) analysis were selected. The thermal profile for all Q-PCR experiments was set (two minutes at 50°C, ten minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C, see figure 2.13); as recommended by manufacturer's instructions.
- For analysis using the MxPro software, HEX and FAM were selected, amplification plots were shown. To analyse results the ROX reference dye was



selected, the fluorescence dye selected was dRN. The Y axis scale was converted to log, thresholds were appropriately adjusted, the text report was exported to excel for tabulating.



**Figure 2.13** The thermal profile set up for the quantitative PCR experiments.

### 2.3.5 Quantitative Taqman® PCR in HASM, HBEC, HMC-1

Expression levels of IL-33, ST2 soluble receptor (ST2s) and ST2 transmembrane receptor (ST2L) mRNA were compared in human mast cell line-1 (HMC-1), human airway smooth muscle (HASM) and human bronchial epithelial cells (HBEC) using quantitative PCR (Mx300SP real-time thermal cycler, Stratagene, La Jolla, California). Many different housekeeping genes expressed both at low and high Ct (threshold cycle) levels were trailed to standardise results. Due to relatively uniform expression in all samples and a similar Ct range, for the results shown, expression from each cDNA sample was normalised to the house keeping

gene HPRT (hypoxanthine-guanine phosphoribosyltransferase) using a pre-designed assay (Applied Biosystems, Foster City, California). All results shown are expressed as percentages relative to either HMC-1 or HBEC expression levels serving as 100% expression. Using Beacon designer 7 (Stratagene), all primer and probe assays were designed to span an intron-exon boundary (to eliminate genomic DNA contamination). Primers were purchased from Invitrogen. The probes were dual labelled with 5' FAM and 3' TAMRA dyes and were purchased from Applied Biosystems, as was the universal master mix and the GAPDH, HPRT and 18S pre-designed assays. Each sample was run in triplicate using 900 nM forward and reverse primers and 250 nM probe. RT- samples and no template controls were analysed in parallel. A standard protocol was used to detect amplification consisting of two minutes at 50°C, ten minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. The list of primers and probes used for comparison of gene expression is provided (table 2.2).

**Table 2.2. Primers and probes used for quantification of mRNA between HMC-1, human airway smooth muscle and human bronchial epithelial cells. PDAR: pre-designed assays, bp: base pairs. N A: data not available. All primers (40 nmol) were purchased from Invitrogen, all probes (40 nmol) were purchased from Applied Biosystems.**

Gene human	Forward primer	Reverse primer	Probe	5' label	3' label	Amplicon	Exons targeted
IL-33	TAGGA GAGAA ACCAC CAAAA GG	GATATA CCAAA GGCAA AGCACT C	CATCT GGTAC TCGCT GCCTG TCAAC A	FA M	TAM RA	117bp	2 - 3 at 197 - 313bp
ST2s	CAAAC AAAAG TATTC CCACT CAGG	ACATTC GCATAT CCAGTC CTATTG	TCGTGT GTTTGC CTCAG GCCAA CT	FA M	TAM RA	148bp	3 - 4 at 492 - 376bp
ST2L	CCTGG AGAAG ATGTA GTCAC TGC	CGTTCT GGATG AGGGC ACAG	AGGGC AACCT CCTGCT CGTAG GCA	FA M	TAM RA	145bp	10 - 11 at 1547 - 1691bp
18S,  GAPD H	PDAR			HEX	N A	N A	N A

### 2.3.6 Quantitative Taqman® PCR analysis

#### 2.3.6.i Initial validation of primer sets

When using the comparative Ct method, it is necessary to ensure that all targets and endogenous controls have similar assay efficiencies. Initially standard curves were set up to determine the assay efficiency, cDNA known to clearly express the target genes were chosen for six cDNA dilutions, decreasing with a two fold difference (1, 0.5, 0.25, 0.125, 0.0625, 0.03125). Each concentration was tested in triplicate, the mean of the three values were plotted against the  $-\log$  of the six cDNA

concentrations (0, -0.3, -0.6, -0.9, -1.2, -1.5). The linear regression slope value was calculated, this value was then used in the equation:  $(10^{(-1/\text{slope value})} - 1) \times 100$  to calculate the primer efficiencies. A slope value of -3.3 equates to 100% primer efficiency. In these experiments all primer efficiencies were of similar values indicating the comparative method could be used for comparison of expression levels between genes.

### 2.3.6.ii Comparative analysis using $\Delta\Delta\text{Ct}$

For each of the three replicates of a sample, the mean cycle time (Ct), mean standard deviation and coefficient of variation were calculated. For each sample the mean Ct of the gene of interest (GOI) was normalised to the mean of the reference gene (ref) (housekeeping gene) for the same sample:  $\Delta\text{Ct} = \text{mean Ct (GOI)} - \text{mean Ct (ref)}$ . After this step a cell sample, or non treatment group was chosen to be the control group to which other samples would be compared (the mean values were calculated from the  $\Delta\text{Ct}$  values generated for these samples to give  $\Delta\text{Ct}$  control values for reference). For example when comparing expression of the GOI in PBS and OVA sensitised lung tissue, means were calculated for all the PBS  $\Delta\text{Ct}$  values, this value generated was then used to calculate the  $\Delta\Delta\text{Ct}$  using the equation:  $\Delta\Delta\text{Ct} = \Delta\text{Ct (sample)} - \Delta\text{Ct (control mean)}$ . Finally the fold difference was calculated relative to the control sample using the equation for each sample: **fold difference** =  $2^{(-\Delta\Delta\text{Ct})}$ .

### **2.3.6.iii Statistics**

All Q-PCR data in Chapter Five, investigating up regulated gene expression in the OVA sensitised lung tissue vs. PBS controls, used the Mann Whitney test for analysis of non-parametric distribution, significance was calculated by ranking the data points within each group and comparing the medians between the two groups using PRISM software Graphpad 5.02. All data was represented as a scatter plot to indicate the variation between individual mice within each group, red lines indicate medians.

For all Q-PCR results designed to look for expression of IL-33, ST2s and the STL receptor in human airway smooth muscle (HASM), bronchial epithelial cells (HBEC) and mast cells (HMC-1), data were compared against a selected cell type and represented as percentage expression.

### **2.3.7 Affymetrix Microarray data**

For microarray results shown in Chapter Five, all data was obtained from a data base generated by Shailandra Singh, TMM (Singh, Billington et al. 2010). Studies aimed to create a gene expression profile using HASM low passage, HASM high passage and lung fibroblast (MRC5) cells. For data shown, screening was performed using Affymetrix oligonucleotide arrays. MRC5 cells were used as the reference cell type. Different housekeeping genes were assessed; results indicated comparatively little variation in expression between cell types. In preparation for the studies, cDNA was synthesised using the GeneChip WT (Whole Transcript) Sense Targeting Labeling and Control reagents kit, following manufacturer's instructions

(Affymetrix, Santa Clara, CA). The sense cDNA was then fragmented by uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE1) and biotin-labeled with terminal deoxynucleotidyl transferase (TdT) using the GeneChip WT Terminal Labeling Kit (Affymetrix). Hybridization was performed by incubation with the Affymetrix GeneChip Human Gene 1.0 ST array; for 28,869 well-annotated genes with 764,885 distinct probes. The arrays were scanned using the GeneChip Scanner 3000 7G (Affymetrix) using GeneChip Operating Software (GCOS). The data generated was analysed using GeneSpring (version 9.0; Agilent Technologies). The Affymetrix data produced can be accessed online at National Center for Biotechnology Information (NCBI) Gene Expression Omnibus using the accession reference GSE18038 (<http://www.ncbi.nlm.nih.gov/geo/>).

The results shown in Chapter Five were generated from data obtained by first searching for each gene of interest in the full Affymetrix data set to locate a corresponding ID reference. ID references for individual genes were then searched in the individual data sets for low passage HASM, high passage HASM and lung fibroblasts to obtain the corresponding robust multi-array average (RMA) signal intensity value. RMA is a normalisation approach which reflects background corrected perfect match intensities for each perfect match cell on every GeneChip. This is generated by summing an estimated probe affinity with an estimated GeneChip-specific log-scale expression value (Bolstad, Irizarry et al. 2003; Irizarry, Hobbs et al. 2003; Hochreiter, Clevert et al. 2006). Results shown were expressed as a % of the HPRT (housekeeping gene) RMA signal intensity value in low passage HASM.

## **CHAPTER THREE RESULTS**

# **OPTIMISATION & PHARMACOLOGICAL CHARACTERISATION OF THE PRECISION CUT LUNG SLICE MODEL FOR MOUSE AND PIG**



### 3. CHAPTER THREE

#### 3.1 INTRODUCTION

To explore the mechanisms involved in airway smooth muscle (ASM) contraction and the pathogenesis of the airway hyper-reactivity seen in asthma, previous studies have either used lung function tests *in vivo* or mechanical studies performed in large airway preparations *ex vivo*, discussed in 1.3. To explore  $\text{Ca}^{2+}$  homeostasis in murine and porcine peripheral airways, this project made use of the precision cut lung slice (PCLS) technique; an intact airway model that allows real time quantification of contraction.

The lung slice technique forms the foundation for further experiments throughout this thesis. Initially the lung slice technique and pharmacological protocols were optimised for mouse to demonstrate the effects of selected agents which could potentially alter ASM tone (table 3.1). These experiments provide a baseline for interpreting the contractile behaviour of the airways in naïve wild type mice and establish the appropriate conditions, concentrations and protocols to use; to serve as a template for future experiments in sensitised mouse models. The PCLS technique was then adapted for pig as a prelude to future human studies. The ultimate goal for this project was to apply the lung slice technique to human. However, human lung tissue is difficult to acquire and when received as surgically removed un-intact lobe segments with many holes, it is difficult to inflate. It was therefore necessary to optimise the lung slice technique in a larger animal such as pig.

In order to explore responses for both mouse and pig distal airways, a range of agents were utilised; the rationale for these is shown in table 3.1.

**Table 3.1. Summary of the agents used in this chapter, mechanisms of action known and reasoning for the conditions selected.** *ACh: acetylcholine; 5-HT: serotonin; HA: histamine; PI: phosphatidylinositol; PLC-β: phospholipase C; IP<sub>3</sub>: inositol triphosphate; IP<sub>3</sub>R: IP<sub>3</sub> receptor; RyR: ryanodine receptor; SERCA pump: sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup>-ATPase pump; ROCC: receptor operated calcium channel; VOCC: voltage operated calcium channels; SOCC: store operated calcium channels; CPA: cyclopiazonic acid; 2-APB: 2-Aminoethoxydiphenyl borate; La<sup>3+</sup>: lanthanum; Ni<sup>2+</sup>: nickel; TRPC: transient receptor potential canonical.*

	Mechanism of action	Reasons for selection	Expected effect on contraction in peripheral airways	References
<b>Acetylcholine (ACh), dose response</b>	ACh mediates smooth muscle contraction through stimulation of the muscarinic M3 receptors which activate the phospholipase C pathway, resulting in IP <sub>3</sub> release. IP <sub>3</sub> then binds to the SR located IP <sub>3</sub> R allowing release of Ca <sup>2+</sup> from internal stores, increasing cytosolic Ca <sup>2+</sup> and leading to contraction.	ACh is a common agonist causing increased airways resistance, used to study the IP <sub>3</sub> pathway. This protocol was designed to establish appropriate dose response ranges in naïve mice.	Previously concentration dependent contractions have been demonstrated in mouse tracheal smooth muscle preparations and in the PCLS model by Bergner and Sanderson.	(Mak and Barnes 1990; Roffel, Elzinga et al. 1990; Bergner and Sanderson 2002; Coulson and Fryer 2003; Berridge 2007)
<b>Serotonin (5-HT), dose response</b>	In many species, excluding human, 5-HT constricts airways via the 5-HT <sub>2</sub> receptor and also increases ACh release from airway nerves. In mice	5-HT plays a role in increased airway resistance and has been implicated in the remodelling process in a murine model of asthma (Lima, Souza et al.	Previous studies in mouse isolated tracheal segments and PCLS suggest 5-HT induces airway smooth muscle contraction.	(Levitt and Mitzner 1989; Barnes 1998; Eum, Norel et al. 1999; Bergner

	the 5-HT induced mechanisms of bronchoconstriction may depend on the cholinergic parasympathetic pathway, as 5-HT induced constriction is blocked by atropine.	2007).		and Sanderson 2002; Moffatt, Cocks et al. 2004; Lima, Souza et al. 2007; Konig, Krain et al. 2009)
<b>Histamine (HA), dose response</b>	Histamine is synthesised and released by mast cells in the airway wall and by circulating basophils. It stimulates PI hydrolysis and increases the concentration of IP <sub>3</sub> in ASM.	HA is shown to mediate anaphylactic shock in animal studies. Inhaled HA causes bronchoconstriction in asthmatic subjects.	Bergner and Sanderson previously demonstrated that no contractions were induced to HA in mouse PCLS.	(Dale and Laidlaw 1910; Curry 1946; Grandordy and Barnes 1987; Barnes 2001; Bergner and Sanderson 2002).
<b>Contraction with 0 mM and 2 mM extracellular Ca<sup>2+</sup></b>	CPA is an inhibitor of the sarcoendoplasmic reticulum Ca <sup>2+</sup> ATPase pump (SERCA pump), which plays a role in maintaining and replenishing internal Ca <sup>2+</sup> stores depleted through agonist stimulation.	This experiment was designed to determine whether SERCA pump inhibition would lead to a passive outward leak of Ca <sup>2+</sup> great enough to stimulate a contraction. In addition I determined whether prolonged internal store Ca <sup>2+</sup> deprivation, induced by a state of 0 mM extracellular Ca <sup>2+</sup> in the presence of a SERCA pump inhibitor, would encourage store operated calcium entry (SOCC) due to stimulation via internal store sensors.	Previous reports in non excitable cells show an increase in [Ca <sup>2+</sup> ] <sub>i</sub> after adding CPA. However, in excitable vascular smooth muscle tissue, addition of SERCA pump inhibitors does not appear to affect tension. It was predicted that airway treatment with SERCA pump inhibitors might lead to a rise in [Ca <sup>2+</sup> ] <sub>i</sub> , however, this would probably not induce contraction; based on the hypothesis that in airway smooth muscle, internal Ca <sup>2+</sup> store refilling regulation is maintained	(Thastrup, Cullen et al. 1990; Low, Gaspar et al. 1991; Henry, 1994; Shima and Blaustein 1992; Henry 1994)

			separately from cytosolic $\text{Ca}^{2+}$ concentrations.	
<b>Caffeine with 0 mM and 2 mM extracellular <math>\text{Ca}^{2+}</math></b>	In cells expressing the ryanodine receptor (RyR), a concentration of 20 mM caffeine opens the RyR allowing $\text{Ca}^{2+}$ release, leading to a transient contraction. Extracellular $\text{Ca}^{2+}$ is required for internal store refilling and release to initiate contraction upon agonist stimulation.	This protocol was designed to demonstrate the role of store refilling and release to induce and maintain airway smooth muscle contractions. Caffeine was chosen for its ability to stimulate the RyR. There is controversy over the role of RyR in controlling $\text{Ca}^{2+}$ release in ASM.	It has previously been shown that agonists can initiate but not maintain airway contraction in $\text{Ca}^{2+}$ free conditions. It was predicted that only an initial transient contraction could be induced in a state of 0 mM extracellular $\text{Ca}^{2+}$ .	(Perez and Sanderson 2005)
<b>ACh and caffeine in 0 mM extracellular <math>\text{Ca}^{2+}</math></b>	Caffeine (20 mM) opens the ER located RyR for $\text{Ca}^{2+}$ release, leading to contraction. ACh targets the pathway activating $\text{IP}_3\text{R}$ release of $\text{Ca}^{2+}$ from the ER.	To determine the effects of caffeine on stimulating and maintaining contractions in the presence of physiological and 0 mM extracellular [ $\text{Ca}^{2+}$ ].	Previous studies in mouse have shown that a transient contraction is induced to ACh in a state of 0 mM $\text{Ca}^{2+}$ . In mouse the ability to induce contraction via caffeine in 0mM and 2 mM $\text{Ca}^{2+}$ is variable; this raises further questions about the ryanodine receptors role. The behaviour of the pig airways in response to ACh and caffeine in the absence of extracellular $\text{Ca}^{2+}$ may help to distinguish receptor roles.	Previous studies in mouse. (Perez and Sanderson 2005)
<b>Effects of CPA on ACh induced contraction</b>	CPA inhibits the SERCA pump, which plays a role in maintaining and replenishing internal $\text{Ca}^{2+}$ stores.	This protocol was designed to show the effects of SERCA pump inhibition (preventing store refilling) on the	SERCA pump inhibition should decrease the amount of $\text{Ca}^{2+}$ taken up to be released for contraction; which was predicted to be	(Thastrup, Cullen et al. 1990; Low, Gaspar et al. 1991; Henry,

		magnitude and maintenance of agonist induced contraction.	displayed as a reduction in magnitude or ability to sustain contraction.	1994; Shima and Blaustein 1992; Henry 1994)
<b>Effects of Nifedipine on ACh induced contraction.</b>	L-type voltage operated calcium channel (VOCC) inhibitor, used in the treatment of hypertension.	To determine whether L-type VOCCs contribute to maintenance of peripheral airway contraction.	Previous studies have suggested that maintenance of contraction is reliant on SOCC or ROCC based on pharmacological tools targeting these pathways. In vascular smooth muscle, nifedipine has shown no relaxant effect on arterioles (Perez and Sanderson 2005).	(Ahmed, Foster et al. 1985; Perez-Vizcaino, Tamargo et al. 1993; Perez and Sanderson 2005)
<b>2-Aminoethoxydiphenyl borate (2-APB), inhibitory dose response</b>	Inhibitor of IP <sub>3</sub> receptors and also SOCC at concentrations greater than 100 µM in which inhibition of SOCC entry through TRPC3, TRPC5,6,7 is suggested.	To determine the significance of the SOCC and IP <sub>3</sub> receptor communication pathways required for Ca <sup>2+</sup> influx and release from internal stores to maintain a contraction.	2-APB inhibits both IP <sub>3</sub> and SOCC mediated increases in intracellular Ca <sup>2+</sup> concentrations; in theory 2-APB should prevent/reduce a sustained contraction stimulated by an agonist acting on the PLC pathway.	(Maruyama, Kanaji et al. 1997; Luo, Broad et al. 2001; Bootman, Collins et al. 2002; Trebak, Bird et al. 2002)
<b>Lanthanum (La<sup>3+</sup>), Nickel (Ni<sup>2+</sup>), inhibitory dose response</b>	Inhibit Ca <sup>2+</sup> influx through SOCC. Lanthanides such as La <sup>3+</sup> and gadolinium (Gd <sup>3+</sup> ) are used in the characterisation of TRPCs. They block TRPCs 1,3,6 in the 100 µM range. La <sup>3+</sup> is shown to potentiate and also inhibit TRPC4 and 5, with potentiation occurring in the	To determine the significance of the SOCC channels in Ca <sup>2+</sup> influx required to maintain contraction.	The selectivity of these drugs is not clearly known. In theory a reduction in contraction should be seen after stimulation though the cholinergic pathway.	(Morgan and Jacob 1994; Zamponi, Bourinet et al. 1996; Lee, Gomora et al. 1999; Schaefer, Plant et al. 2000; Luo, Broad et al.

	<p>10-100 <math>\mu\text{M}</math> range.</p> <p><math>\text{Ni}^{2+}</math> is a <math>\text{Ca}^{2+}</math> channel binding inhibitor. Only one mammalian T-type <math>\text{Ca}^{2+}</math> channel, <math>\text{Ca}_v3.2</math> is strongly inhibited by <math>\text{Ni}^{2+}</math>. <math>\text{Ni}^{2+}</math> works in part by occlusion of the pore and is also shown to bind to a site on the extracellular side of the channel, affecting current through the channel allosterically, demonstrated in neuronal <math>\text{Ca}^{2+}</math> channels.</p>			<p>2001; Putney 2001; Trebak, Bird et al. 2002; Jung, Muhle et al. 2003; Dietrich, Mederos y Schnitzler et al. 2005)</p>
<p><b>Icrac inhibitors:</b>  <b>JS (GSK1349571A)</b>  <b>N117 (N11764-14-1),</b>  <b>inhibitory dose response,</b>  <b>effects of Icrac inhibition in SOCC</b>  <b>favoured conditions</b></p>	<p>Mechanism of action unknown, compounds were acquired for testing from GlaxoSmithKline. Activation of the <math>\text{Ca}^{2+}</math> release activated <math>\text{Ca}^{2+}</math> channel (CRAC) current is initiated by STIM proteins, which sense ER <math>[\text{Ca}^{2+}]</math> and upon store depletion, relocate to form puncta closely associating with the plasma membrane pore forming channel subunit of the CRAC channel, Orai.</p>	<p>Inhibition of <math>\text{Ca}^{2+}</math> influx through this channel in physiological and SOCC induced conditions should highlight the importance of SOCC in maintaining contraction.</p>	<p>Studies focus on the use of Icrac inhibitors in T cells and the immune response. Using JS ((GSK1349571A) also referred to as Synta 66) inhibition of CRAC channel function decreased the T cell response in lamina propria mononuclear cells of inflammatory bowel disease patients. GSK has previously reported inhibition of OVA-induced contractions of rat trachea and allergen evoked contractions of human bronchus (through mast cell degranulation). No studies</p>	<p>(Feske, Gwack et al. 2006; Prakriya, Feske et al. 2006; Yeromin, Zhang et al. 2006; Di Sabatino, Rovedatti et al. 2009)</p>

			have been cited for the effects of these Icrac inhibitors on skeletal or smooth muscle tone.	
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### **3.1.1 AIMS**

The aim of the work described in this chapter was to establish a precision cut lung slice model for mouse and pig to provide a platform for investigating agents that alter smooth muscle tone and the signalling pathways that mediate contraction. The rationale for studying specific agents is shown in table 3.1.

## **3.2 METHODS**

Detailed methods for the preparation of the lung slices for mouse and pig, use of an oscillating tissue slicer, measuring contractile response, staining of slices and statistical analysis used are provided in section 2.1 and form the foundation for most experiments throughout this thesis. A range of different chemical perfusion protocols were used for the experiments in this chapter and are shown with the corresponding airway contraction results throughout the chapter. All chemicals were perfused at a constant flow rate (2 ml/minute) over the lung slice during real time video image capturing of the airway contraction.

### **3.2.1 Statistical analysis**

All data shown in this chapter for airway contraction experiments are derived from the raw values measured for individual airway lumen area ( $\mu\text{m}^2$ ) and manipulated to show percentages relative to the maximum contraction induced by ACh (either 1 or 10  $\mu\text{M}$ ) and the initial relaxed airway measurements (baseline) (see 2.1.5). For all results shown, at least three airways from three WT C57BL/6 mice

and three airways from three WT domesticated pigs were used. Dose response experiments were analysed with PRISM software Graphpad 5.02 using analysis tools for dose response for the log of the agonist against the response for variable data;  $EC_{50}$  and  $IC_{50}$  measurements with confidence intervals (CI) are provided, error bars indicate standard deviation (SD). The  $EC_{50}$  (referring to agonist) or  $IC_{50}$  (referring to inhibitor) is defined as the concentration of drug that provokes a response halfway between the baseline and maximum response. This measure helped me to determine the concentration range and potency of the drugs investigated. All other data are shown as scatter plots; each dot represents an individual mouse or pig airway. Blue lines indicate the mean value within each group. For these studies all slices came from naïve WT lung tissue and the same slice was exposed to each condition throughout the protocol. Therefore, data in this chapter were analysed using one-way ANOVA followed by the Dunnett's post test comparing means within treatment (drug) groups to the mean of the control (buffer) group. The underlying mathematics is the same for a T-test however, designed for three or more treatment groups.

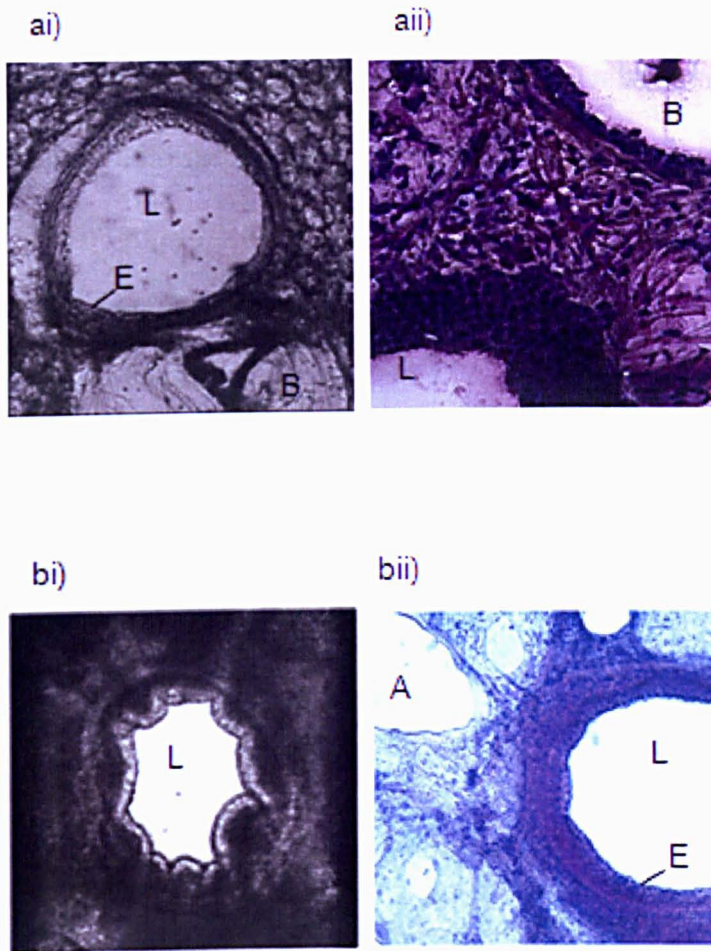
### 3.3 RESULTS

Approximately 300 slices were obtained from each set of mouse lungs for experiments. Slices used for mouse contractile experiments were 130  $\mu\text{m}$  thick, slices used for haematoxylin and eosin (H & E) staining were 20  $\mu\text{m}$  thick. For each segment of pig lung tissue inflated (average lung segment volume of 35 mls), 300 slices were cut. Pig slices used for contractile experiments had a thickness of 140 - 170  $\mu\text{m}$ , slices used for H & E staining were 75  $\mu\text{m}$  thick. Only distal airways were used for experiments (airway diameter 100 - 300  $\mu\text{m}$ ). For contractile experiments airways with beating cilia and a lumen free from agarose were chosen. An initial test contraction with 10  $\mu\text{M}$  ACh was performed to ensure airways were capable of generating a contractile response; only airways with a reduction in lumen area of > 25% were used for the studies. Leaving each drug condition perfusing for five minutes minimum was enough time to allow the airway to react accordingly (relax or contract) before changing the conditions or altering the concentration for the next step of the protocol. ~90% of the maximum contraction to ACh was achieved within four minutes of perfusion, however, further contraction was observed with prolonged agonist stimulation (as shown in figure 3.2). Due to protocol time constraints, to obtain 'maximum airway contraction measurements'; airways were perfused with ACh for five minutes and values for contraction level were taken at the end of this period. For store operated  $\text{Ca}^{2+}$  inhibitor airway relaxation experiments (section 3.3.6), ACh was present initially to contract the airway and maintained for 35 minutes. Even in the presence of the inhibitors the degree of contraction often increased beyond that measured in the first five minutes of exposure to ACh. Airway behaviour showing further contraction or failure to relax to base line levels was also seen (see section 3.3.4) for airways exposed to abnormal extracellular  $\text{Ca}^{2+}$

conditions. Previous experiments indicated that airway relaxation in mouse and pig was achieved within five - seven minutes of perfusion with wash buffer. After contraction to a maximum concentration of agonist, 90% of airways relaxed (indicated by a plateau after relaxation movement). Therefore in all experiments a ten minute wash buffer perfusion phase was used to allow airway relaxation. The slices were kept in media supplemented with glutamine, penicillin and streptomycin, the media was changed after four days (see section 2.1). Airways still produced a contractile response on the six<sup>th</sup> day; however, the airways did not maintain their contractile response and showed 'twitching' making airway tracings difficult to interpret. In view of this, for the experiments performed in this thesis, slices were used one - three days after preparation. In some airways variability in degree of contraction, relaxation or baseline drift occurred (reflected in results).

### **3.3.1 Images of mouse and pig airways**

Mouse airways chosen for experiments were circular in shape (elongated airway shapes were avoided). Images captured in real time required either 100x or 200x magnification, for airways of 100 - 150  $\mu\text{m}$  in diameter. Mouse airway lumen areas ranged from 16,000  $\mu\text{m}^2$  to 100,000  $\mu\text{m}^2$ . Pig peripheral airway structure differs anatomically from mouse (discussed in section 3.4.1), airways are circular or weblike in shape. Airways chosen for analysis in pig had a diameter of 100 - 150  $\mu\text{m}$  and initial airway lumen areas ranging from 10,000  $\mu\text{m}^2$  to 100,000  $\mu\text{m}^2$ .



**Figure 3.1 Mouse and pig peripheral airways, bright field and H&E images.** L: airway lumen; B: blood vessel; E: epithelium; A: alveolus.

ai) Representative image of a WT C57BL/6 mouse airway slice of 130  $\mu\text{m}$  thickness at 100x magnification with a diameter of  $\sim 100 \mu\text{m}$ ; beating cilia were visible by eye on the epithelial lining during live imaging.

aii) Representative image for H & E staining of a WT C57BL/6 mouse lung slice of 20  $\mu\text{m}$  thickness taken at 200x magnification.

bi) Representative image of a pig airway slice of 140  $\mu\text{m}$  thickness, at 100x magnification with a diameter of  $\sim 100 \mu\text{m}$ , beating cilia were visible on the epithelial lining

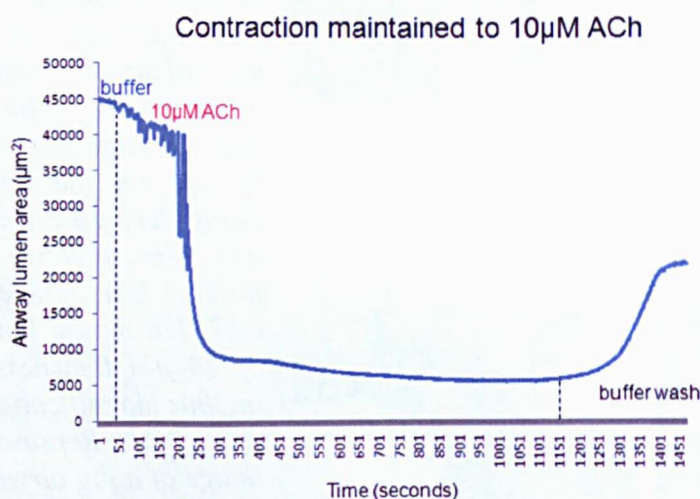
during live imaging. bii) Representative image for H & E staining of a pig peripheral airway slice at 75  $\mu\text{m}$  thickness, taken at 100x magnification.

### 3.3.2 Differences in mouse and pig contractile response to acetylcholine, serotonin and histamine

For the following results shown, PCLS were used from at least three C57BL/6 WT mice and three domestic pigs to examine contractile responses. For each airway, experiments were performed to assess contractile responses to ACh (10  $\mu\text{M}$ ) prior to further studies. The following graph (figure 3.2) shows that the airway contraction is maintained with stimulation of ACh over a prolonged period of time (20 minutes) before buffer wash out to relaxed state (results consistent in both mouse



(n = ten) and pig (n = ten) data not shown). This indicates a prolonged state of contraction can be maintained with agonist stimulation present.



**Figure 3.2 ACh induced contraction is maintained until wash out.** Example of an original airway tracing (representative of n = ten airways, ten C57BL/6 mice), the contraction is induced (88.0% contraction compared to relaxed airway at buffer perfusion) and maintained by ACh for 20 minutes. The airway only partially relaxed after ten minutes of perfusion with

agonist free buffer wash. This experiment shows an initial maximum contraction followed by a sustained phase which requires extracellular  $\text{Ca}^{2+}$  to maintain the airway contraction; as demonstrated throughout this chapter.

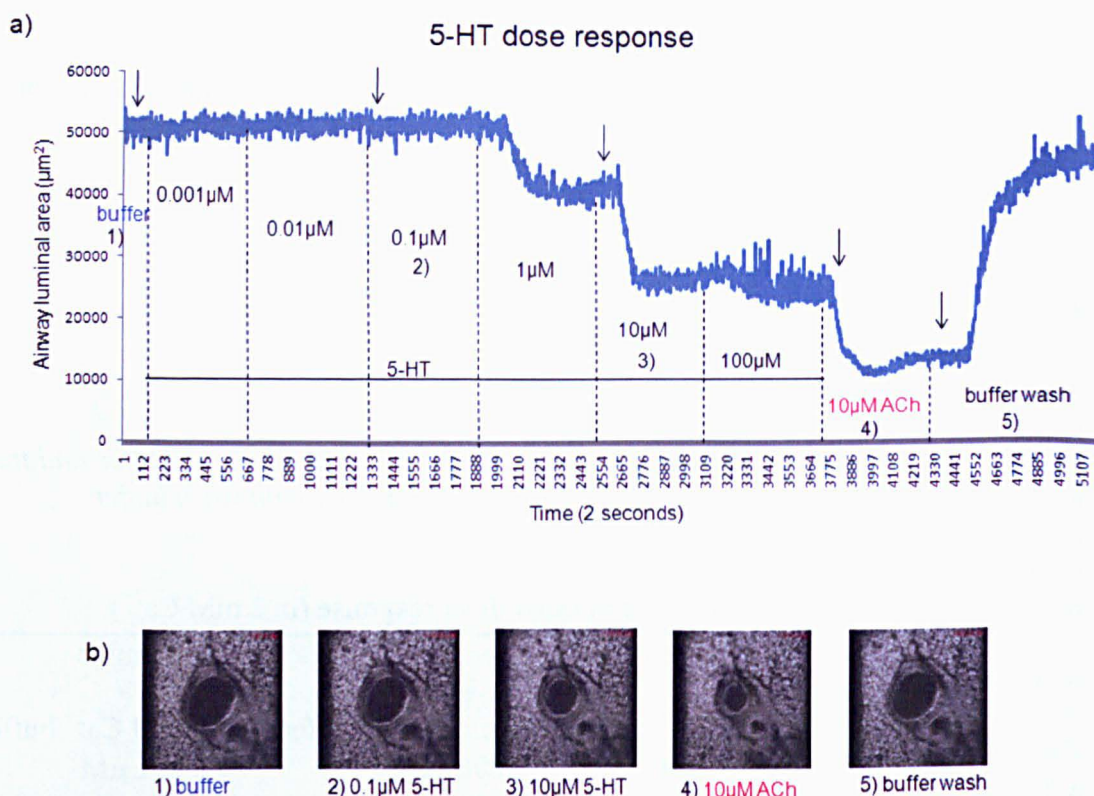
The following agonist dose response protocols for acetylcholine (ACh), serotonin (5-HT) and histamine (HA) were designed to rank agonist potency and provide a comparison for distal airway behaviour between mouse and pig. Results are compared to published data using different techniques and species. Differences in airway behaviour between airway generations of the same species were also considered. For the following experiments all buffers and agonists contained 2 mM (physiological) extracellular  $\text{Ca}^{2+}$ . Individual lung slices were perfused with buffer for one minute (image capture of relaxed airway), followed by five minute intervals of increasing concentrations of the agonist (maximum concentration 100  $\mu\text{M}$ ). A final ten minute wash relaxed the airway. In 5-HT and HA protocols, 10  $\mu\text{M}$  ACh perfusion was included for comparison of magnitude of contraction. Perfusion

system timings are shown below (table 3.2). A representative example of an individual airway contraction tracing throughout the experiment is shown for each agonist, followed by graphed data (three airways from three mice, or three airways from three pigs, figures: 3.3, 3.4, 3.5, 3.6, 3.7, 3.8).

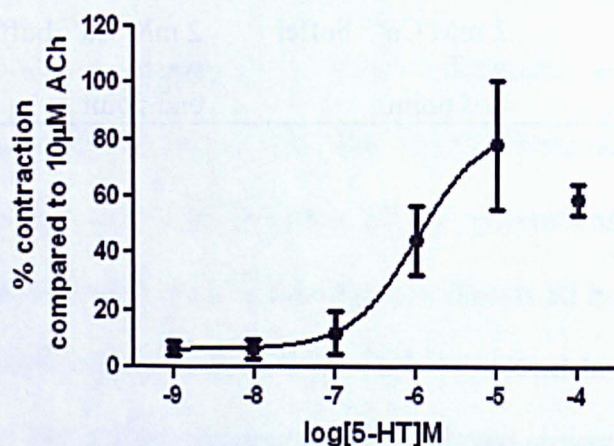
*Table 3.2. Airway agonist dose response perfusion timings for buffer and increasing agonist concentrations until relaxation of the airway with wash buffer.*

<b>Contractile agonist dose response (in 2 mM Ca<sup>2+</sup>)</b>			
<b>Time (minutes)</b>	<b>Acetylcholine (ACh)</b>	<b>Serotonin (5-HT)</b>	<b>Histamine (HA)</b>
0	2 mM Ca <sup>2+</sup> buffer	2 mM Ca <sup>2+</sup> buffer	2 mM Ca <sup>2+</sup> buffer
1	0.0001 µM	0.001 µM	0.001 µM
6	0.001 µM	0.01 µM	0.01 µM
11	0.01 µM	0.1 µM	0.1 µM
16	0.1 µM	1 µM	1 µM
21	1 µM	10 µM	10 µM
26	10 µM	100 µM	100 µM
31	2 mM Ca <sup>2+</sup> buffer	10 µM ACh	10 µM ACh
	wash		
	end point (41 minutes)		
36		2 mM Ca <sup>2+</sup> buffer	2 mM Ca <sup>2+</sup> buffer
46		wash	wash
		end point	end point



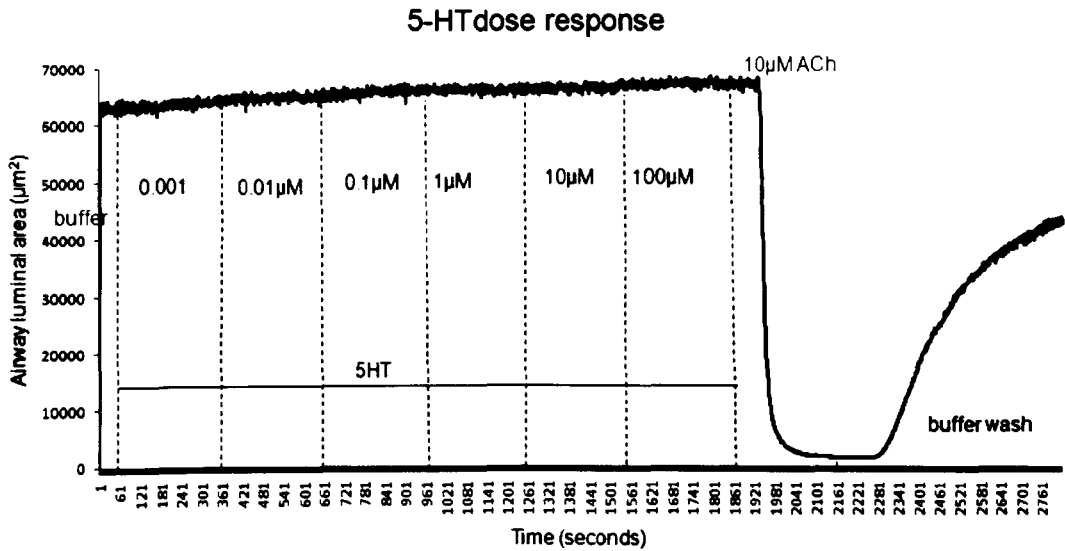


**Figure 3.3a 5-HT induces a concentration dependent contraction in mouse peripheral airways.** a) an example of an airway lumen area tracing for contractile responses to 5-HT ( $10^{-9}$ - $10^{-4}$  M) from a 130  $\mu\text{m}$  thick C57BL/6 mouse lung slice (representative of  $n =$  three airways from three mice). The arrows shown on the graph correspond to the images of the airways in panel b). The maximum contraction induced to 5-HT 100  $\mu\text{M}$  is 68.8% of the contraction induced by ACh.



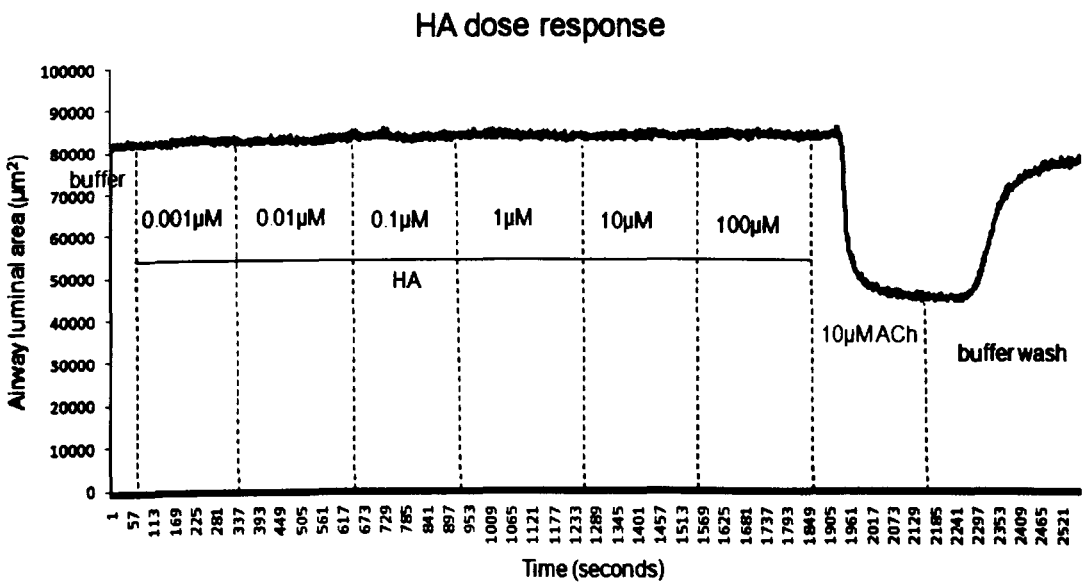
**Figure 3.3b 5-HT induces a concentration dependent contraction in mouse peripheral airways.** Concentration dependent contractions to 5-HT in C57BL/6 mouse lung slices ( $10^{-9}$ - $10^{-4}$  M), ( $EC_{50} = 1.1$   $\mu\text{M}$ ,  $CI = 289.7$  nM - 3.8  $\mu\text{M}$ ). The mean maximum response to 5-HT (10  $\mu\text{M}$ ) was  $77.9 \pm 13\%$ , of the response to 10  $\mu\text{M}$  ACh (100%). At concentrations

of 5-HT  $> 10$   $\mu\text{M}$  a reduction in the degree of contraction was observed. Each data point represents the mean value for  $n =$  three mice, one airway each. Error bars indicate SD of values.  $EC_{50}$  values were calculated in PRISM Graphpad 5.02 using log of agonist vs. response for variable slope (for logM concentrations from -9 to -5).



**Figure 3.4** 5-HT does not induce contraction in pig peripheral airways. An airway lumen area tracing in pig peripheral airways showing no contractile response to 5-HT (representative of  $n = \text{three}$ , three pigs). A control response is shown to 10  $\mu\text{M}$  ACh.

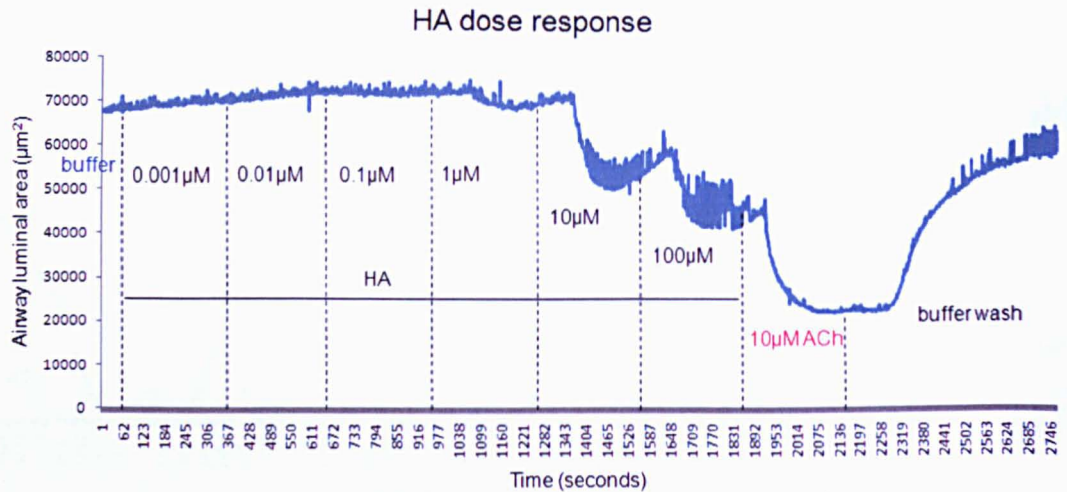
As 5-HT failed to elicit a response in pig airways, mean results for  $n = \text{three}$  airways have been omitted.



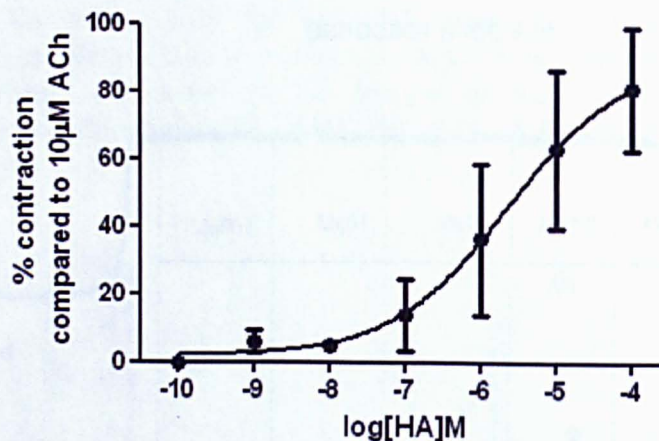
**Figure 3.5** HA does not induce a contractile response in mouse peripheral airways. An example of an airway lumen area tracing in a C57BL/6 mouse lung slice (130  $\mu\text{m}$  thick, representative of  $n = \text{three}$ , three mice) showing no contractile response to HA; a control response to 10  $\mu\text{M}$  ACh is shown.

As HA failed to induce a contractile response in mouse airways, mean results have been omitted.



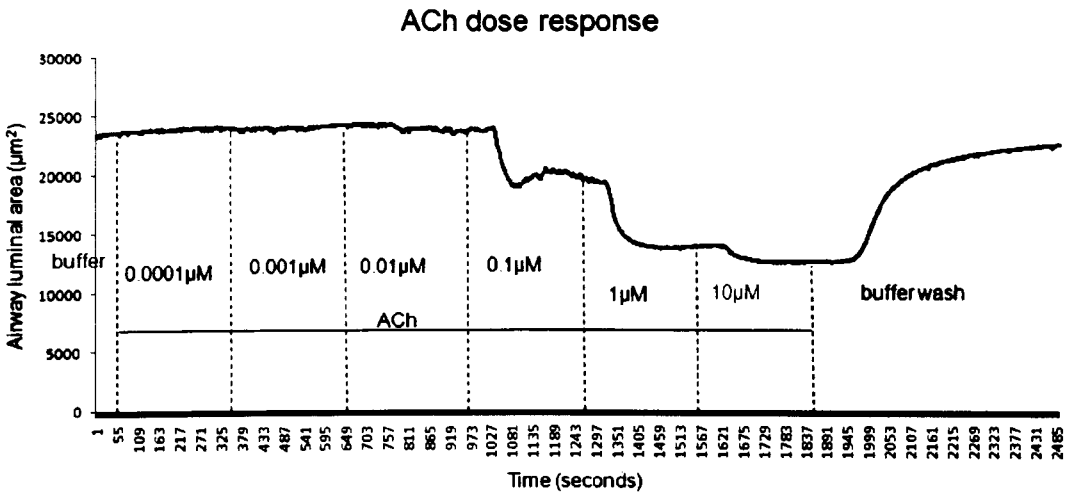


**Figure 3.6a** HA induces a concentration dependent contraction in pig peripheral airways. An example of an airway lumen area tracing for contractile responses to HA ( $10^{-9}$  -  $10^{-4}$  M) in a  $170 \mu\text{m}$  thick lung slice (representative of  $n =$  three airways, three pigs). In some tracings relaxation was observed after initial contraction to a set concentration (shown on the graph at  $10 \mu\text{M}$  and  $100 \mu\text{M}$  HA).

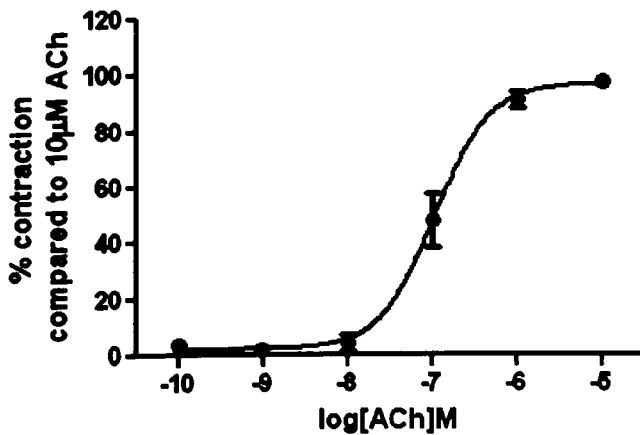


**Figure 3.6b** HA induces a concentration dependent contraction in pig peripheral airways. Concentration dependent contractions were observed ( $10^{-9}$  -  $10^{-4}$  M) ( $\text{EC}_{50} = 2.2 \mu\text{M}$  ( $\text{CI} = 230.2 \text{ nM} - 21.7 \mu\text{M}$ )). The maximum mean contractile response to HA was  $80.3\% \pm 29.5\%$  induced by  $100 \mu\text{M}$  HA in comparison to  $10 \mu\text{M}$  ACh

(100% contraction). Each data point represents the mean value for  $n =$  three airways, three pigs. Error bars indicate SD.  $\text{EC}_{50}$  values were calculated in PRISM Graphpad 5.02 using log of agonist vs. response for variable slope.

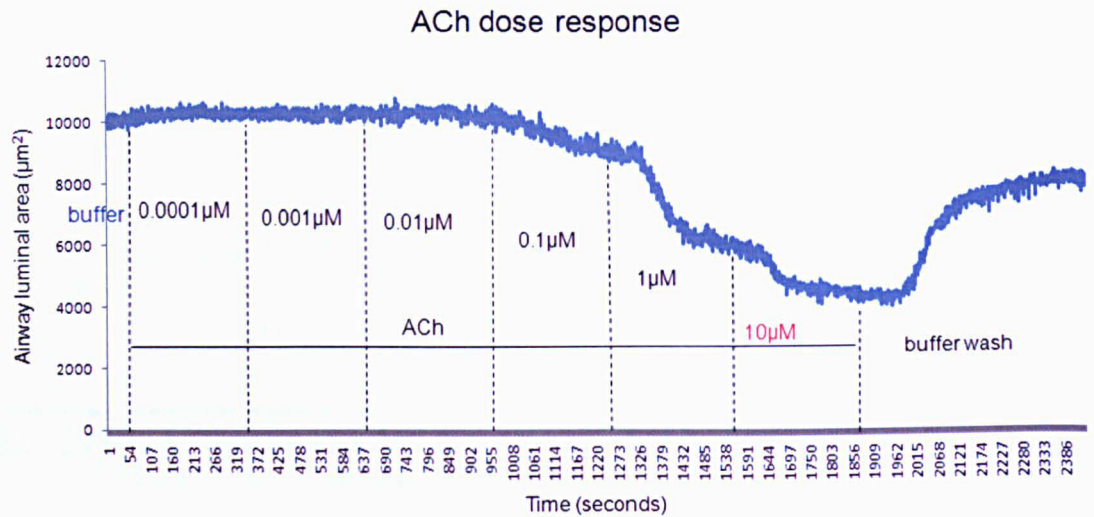


**Figure 3.7a** ACh induces concentration dependent contractions in mouse peripheral airways. A representative tracing of an ACh induced contractile response ( $10^{-10}$ - $10^{-5}$  M) in a WT C57BL/6 mouse lung slice (representative of  $n =$  three airways, three mice).

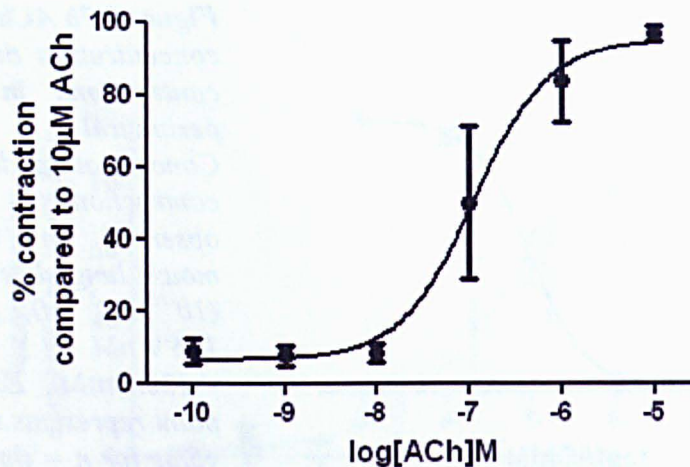


**Figure 3.7b** ACh induces concentration dependent contractions in mouse peripheral airways. Concentration dependent contractions were observed in C57BL/6 mouse lung slice airways ( $10^{-10}$ - $10^{-5}$  M), ( $EC_{50} = 106.9$  nM,  $CI = 87.7$  nM - 130.3 nM). Each data point represents the mean value for  $n =$  three mice, one airway each. Error

bars indicate SD.  $EC_{50}$  values were calculated in PRISM Graphpad 5.02 using log of agonist vs. response for variable slope.



**Figure 3.8a ACh induces concentration dependent contraction in pig peripheral airways.** A representative tracing of an ACh ( $10^{-10}$ - $10^{-5}$  M) contractile response in a pig lung slice peripheral airway ( $n = \text{three}$ , three pigs).

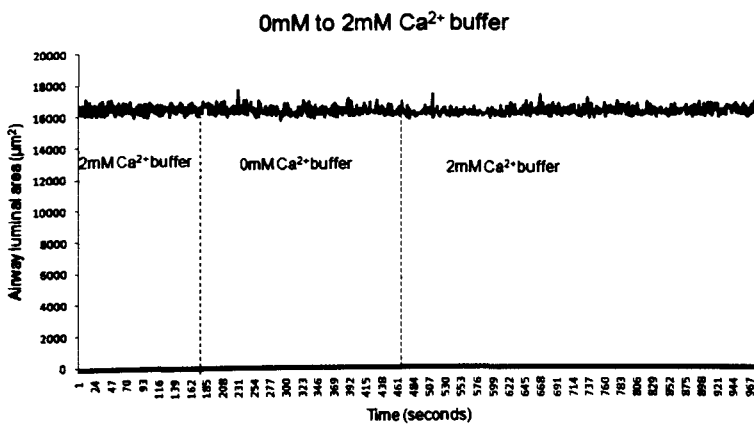


**Figure 3.8b ACh induces concentration dependent contraction in pig peripheral airways.** Concentration dependent contractions were observed to ACh in pig lung slices ( $10^{-10}$ - $10^{-5}$  M).  $EC_{50} = 112.5$  nM (CI = 59.7 nM - 211.9 nM). Each data point represents the mean value for  $n = \text{three}$  airways, three pigs. Error bars indicate SD of

values.  $EC_{50}$  values were calculated in PRISM Graphpad 5.02 using log of agonist vs. response for variable slope. The graph does not initially start at 0% contraction, due to airway relaxation.

### 3.3.3. Significance of extracellular $\text{Ca}^{2+}$ and store released $\text{Ca}^{2+}$ in initiating and maintaining a contraction

The following experiments were designed to explore the requirement of extracellular  $\text{Ca}^{2+}$  for i) intracellular store refilling and ii) the direct role of store released  $\text{Ca}^{2+}$  for further contraction induction and maintenance. Initial experiments were performed under unstimulated (i.e. no agonist) conditions to investigate the effect of changing from 2 mM (physiological) extracellular  $\text{Ca}^{2+}$  to 0 mM extracellular  $\text{Ca}^{2+}$  (figure 3.3.3.0). As can be seen, neither the change to 0 mM  $\text{Ca}^{2+}$  nor return to 2 mM  $\text{Ca}^{2+}$  altered airway calibre (also performed in pig, n = three, results not shown).



**Figure 3.9** *No contraction is induced with the readdition of  $\text{Ca}^{2+}$ . Airway tracing in a 130  $\mu\text{m}$  thick, C57BL/6 mouse lung slice (representative of n = three, three mice) showing initial perfusion with 2 mM  $\text{Ca}^{2+}$  buffer followed by 0 mM  $\text{Ca}^{2+}$  buffer.*

*No alteration in calibre of the airway was induced by reintroducing extracellular  $\text{Ca}^{2+}$  (2 mM) after perfusion with 0 mM  $\text{Ca}^{2+}$  buffer.*

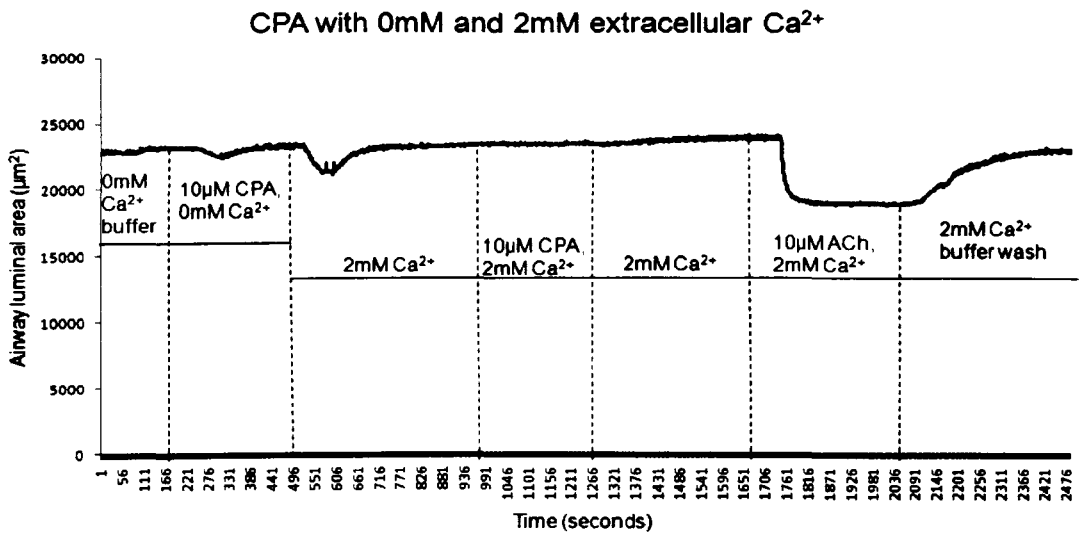
Having demonstrated that altering extracellular  $\text{Ca}^{2+}$  by itself does not alter airway calibre in PCLS, I next explored the effect of depleting the intracellular  $\text{Ca}^{2+}$  store by the use of the SERCA inhibitor cyclopiazonic acid (CPA) in the presence of 0 mM extracellular  $\text{Ca}^{2+}$ . Prior to experiments the slices were kept in 2 mM  $\text{Ca}^{2+}$  buffer. The perfusion protocol (table 3.3) involved initial perfusion of 0 mM  $\text{Ca}^{2+}$  followed by CPA (to prevent  $\text{Ca}^{2+}$  SERCA pump uptake into SR stores), in addition

passive outward leak of  $\text{Ca}^{2+}$  from internal stores should be occurring at this time. This store depletion should trigger SOCC for  $\text{Ca}^{2+}$  entry. At eight minutes 2 mM  $\text{Ca}^{2+}$  was perfused, to determine whether a contraction would be seen at this point due to activated SOCC and replenish of internal stores for  $\text{Ca}^{2+}$  release. CPA in 2 mM  $\text{Ca}^{2+}$  was perfused to show that with physiological extracellular  $\text{Ca}^{2+}$  conditions (2 mM) SERCA pump inhibition does not lead to a contraction. This was followed by a control contraction to 10  $\mu\text{M}$  ACh for analysis comparison and finally a wash with buffer. A corresponding representative airway contraction tracing and collective results for mouse (three airways from three mice) and pig (six airways from three pigs) are shown (figures: 3.10, 3.11).

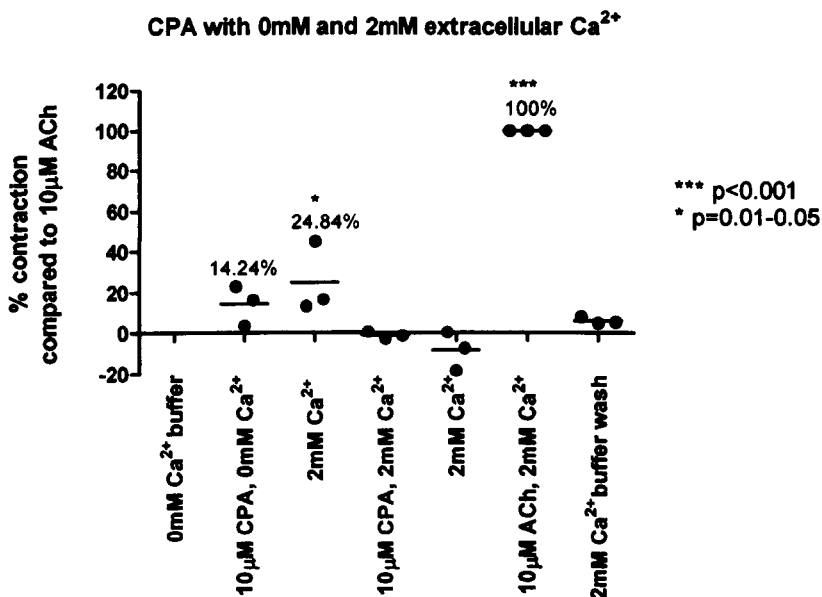
*Table 3.3 Perfusion timings designed to show the influence of altering extracellular  $\text{Ca}^{2+}$  concentrations on inducing SOCC entry and SR release for airway contraction (described above in text).*

<b>CPA with 0 mM and 2 mM extracellular <math>\text{Ca}^{2+}</math></b>	
<b>Time (minutes)</b>	<b>Perfusion</b>
0	0 mM $\text{Ca}^{2+}$ buffer
3	10 $\mu\text{M}$ CPA, 0 mM $\text{Ca}^{2+}$
8	2 mM $\text{Ca}^{2+}$
13	10 $\mu\text{M}$ CPA, 2 mM $\text{Ca}^{2+}$
18	2 mM $\text{Ca}^{2+}$
23	10 $\mu\text{M}$ ACh
28	2 mM $\text{Ca}^{2+}$ buffer wash
38	end point



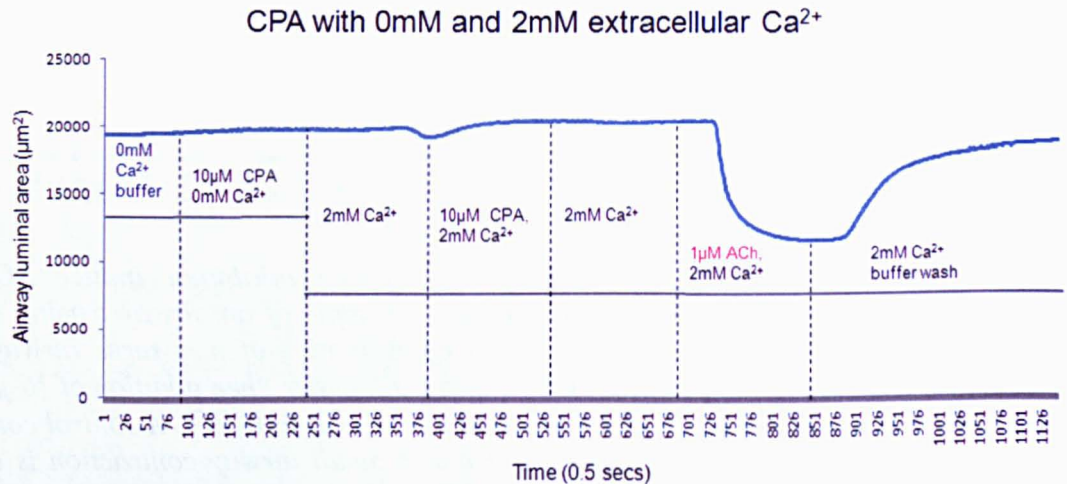


**Figure 3.10a** 0 mM  $\text{Ca}^{2+}$  and SERCA pump inhibition induce SOCC for contraction in mouse peripheral airways. Example of an airway tracing in a 130  $\mu\text{m}$  thick, C57BL/6 mouse lung slice (representative of  $n = \text{three airways, three mice}$ ) showing initial perfusion with 0 mM  $\text{Ca}^{2+}$  buffer, then addition of 10  $\mu\text{M}$  CPA, followed by 2 mM  $\text{Ca}^{2+}$  buffer then 10  $\mu\text{M}$  CPA in 2 mM  $\text{Ca}^{2+}$ . A control contraction is shown to ACh prior to buffer wash out. A small airway contraction is observed following  $\text{Ca}^{2+}$  readdition. In contrast, following CPA exposure in 2 mM  $\text{Ca}^{2+}$ , 2 mM extracellular  $\text{Ca}^{2+}$  alone failed to elicit a response.

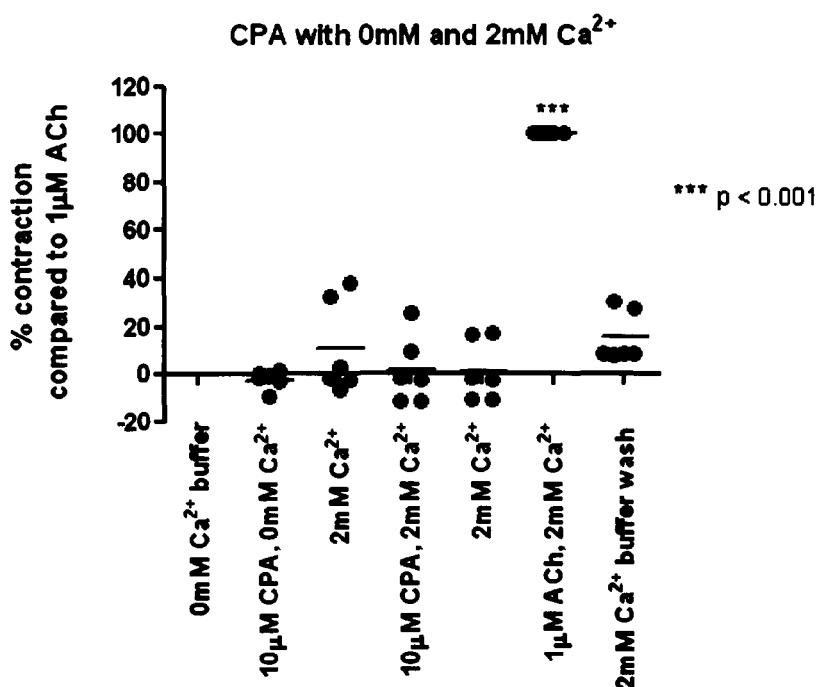


**Figure 3.10b** 0 mM  $\text{Ca}^{2+}$  and SERCA pump inhibition induce SOCC for contraction in mouse peripheral airways. Contractile responses in WT C57BL/6 mouse lung slice airways ( $n = \text{three mice}$ ), normalised to 0 mM  $\text{Ca}^{2+}$  buffer alone as relaxed and 10  $\mu\text{M}$  ACh in 2 mM  $\text{Ca}^{2+}$  as 100% contracted. Data is analysed using one-way ANOVA followed by the Dunnett's post test comparing all means to 0 mM  $\text{Ca}^{2+}$  buffer values, \*\*\*  $p < 0.001$ , \*  $p = 0.01 - 0.05$ . Individual dots represent 1 airway from an individual mouse, blue lines indicate means. Although a small

contraction was seen with the introduction of CPA in 0 mM  $\text{Ca}^{2+}$  (mean = 14.2% contraction) this was not significant in comparison to 0 mM  $\text{Ca}^{2+}$  values (mean = 0). The addition of 2 mM  $\text{Ca}^{2+}$  significantly reduced airway lumen area ( $p = 0.01 - 0.05$ , mean = 24.8% contraction). A significant airway contraction was seen to 10  $\mu\text{M}$  ACh in 2 mM  $\text{Ca}^{2+}$  as indicated,  $p < 0.001$ .



**Figure 3.11a 0 mM  $\text{Ca}^{2+}$  and SERCA pump inhibition for SOCC activation does not induce significant contraction in pig peripheral airways.** Example of an airway tracing in a 170  $\mu\text{m}$  thick pig lung slice (representative of  $n = \text{six}$  airways, three pigs) showing initial perfusion with 0 mM  $\text{Ca}^{2+}$  buffer, then addition of 10  $\mu\text{M}$  CPA, followed by 2 mM  $\text{Ca}^{2+}$  buffer (a very small contraction was seen) then 10  $\mu\text{M}$  CPA in 2 mM  $\text{Ca}^{2+}$ . A control contraction is shown to ACh prior to buffer wash out.



**Figure 3.11b** 0 mM  $\text{Ca}^{2+}$  and SERCA pump inhibition for SOCC activation does not induce significant contraction in pig peripheral airways. Contractile responses in pig lung slice airways ( $n = \text{six airways, three pigs}$ ), normalised to 0 mM  $\text{Ca}^{2+}$  buffer alone as relaxed and 10  $\mu\text{M}$  ACh in 2 mM  $\text{Ca}^{2+}$  as 100% contracted. Data is analysed using one-way ANOVA followed by the Dunnett's post test comparing all means to 0 mM  $\text{Ca}^{2+}$  buffer values, \*\*\*  $p < 0.001$ , \*  $p = 0.01 - 0.05$ . Individual dots represent 1 airway from an individual mouse, blue lines indicate means. Little or no contraction was seen with the introduction of CPA in 0 mM  $\text{Ca}^{2+}$  and the addition of 2 mM  $\text{Ca}^{2+}$  (mean = 10.2% contraction), this was not significant in comparison to 0 mM  $\text{Ca}^{2+}$  values. A significant airway contraction was seen to 10  $\mu\text{M}$  ACh in 2 mM  $\text{Ca}^{2+}$  as indicated,  $p < 0.001$ .

Results show that using the same protocol the readdition of 2 mM  $\text{Ca}^{2+}$  produced a significant contraction in mouse; this contraction was not significant in pig. Although there were small sample sizes, these results hint at differences between mouse and pig in sensitivity to CPA, or SR store regulation required for release of  $\text{Ca}^{2+}$  for contraction.

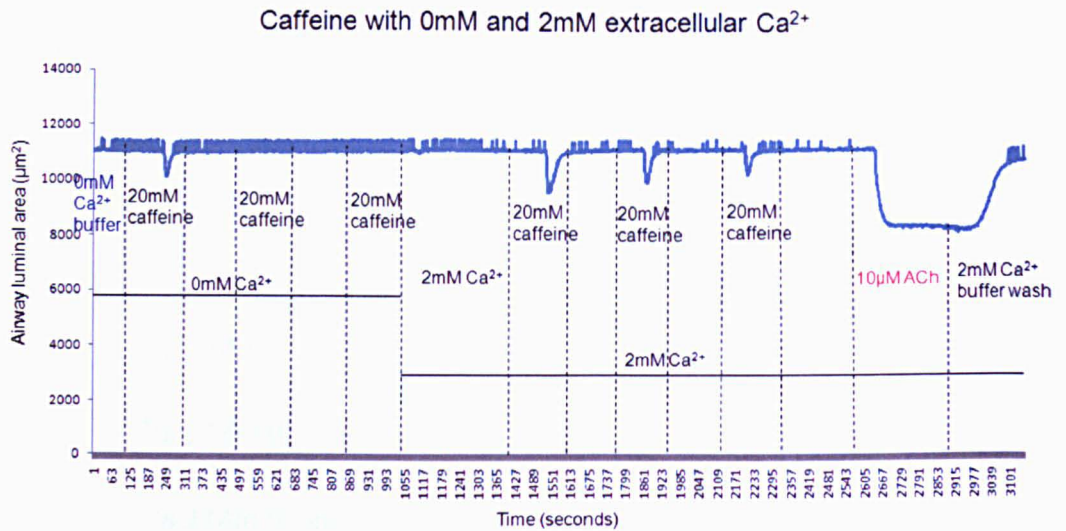
In ASM contraction involves agonist induced mobilisation of  $\text{Ca}^{2+}$  from the SR. This process occurs through a GPCR receptor-mediated pathway that frees inositol-1,4,5-trisphosphate ( $\text{IP}_3$ ) which in turn acts on the  $\text{IP}_3$  receptor located on the

SR to release  $\text{Ca}^{2+}$  leading to downstream contraction of the smooth muscle (discussed in detail in section 1.2) (Berridge 1993; Flynn, Bradley et al. 2001; Dietrich, Chubnov et al. 2006). Another SR located  $\text{Ca}^{2+}$  channel is the ryanodine receptor (RyR). Cyclic adenosine diphosphate ribose (cADPR) is suggested to be the endogenous ligand (Tazzeo, Zhang et al. 2008; Flynn, 2001). The role of the RyR in ASM remains unclear, although indicates involvement in regulating  $[\text{Ca}^{2+}]_i$  within the cytosolic space between the plasmalemma and the SR (Tazzeo, Zhang et al. 2008).

The next experiment was designed to investigate if internal  $\text{Ca}^{2+}$  store refilling for  $\text{Ca}^{2+}$  release is required to induce airway contractions. Caffeine was used to deplete the internal stores by opening (at a concentration of 20 mM) of the ryanodine receptor. Perfusion timings were designed to show airway contractions intermittently induced by caffeine in conditions of 0 mM and 2 mM extracellular  $\text{Ca}^{2+}$  (see table 3.4). Representative airway contraction tracings throughout the protocol are shown for mouse and pig (figure 3.12 and 3.13).

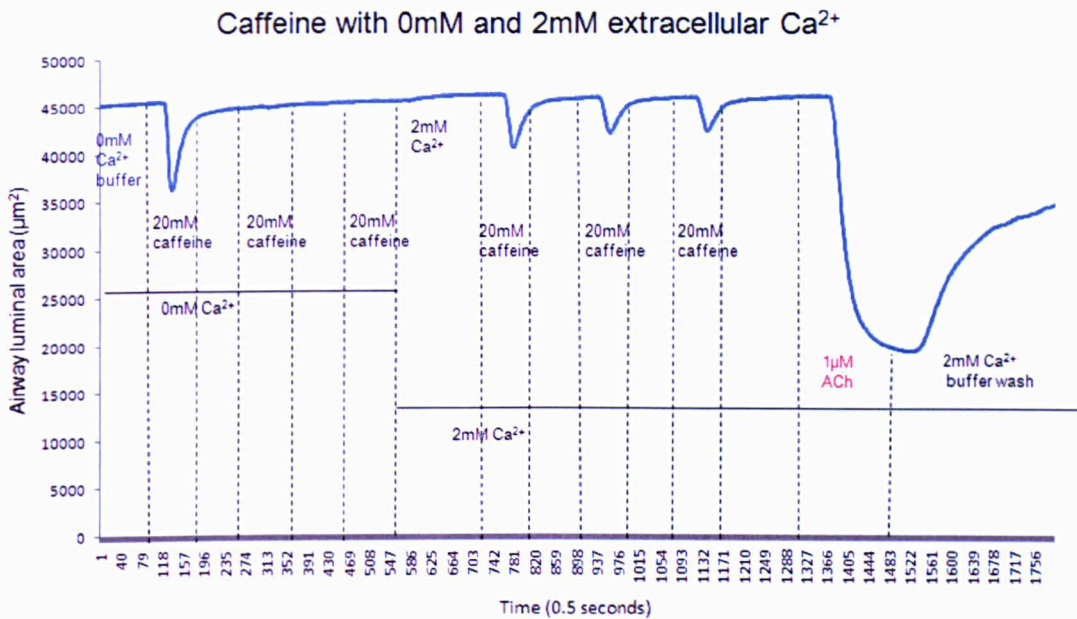
*Table 3.4 Perfusion system timings designed to show that extracellular  $\text{Ca}^{2+}$  is required for internal  $\text{Ca}^{2+}$  store release (using caffeine stimulated ryanodine receptor release); to induce airway smooth muscle contraction.*

<b>Caffeine with 0 mM and 2 mM extracellular <math>\text{Ca}^{2+}</math></b>	
Time (minutes)	Perfusion
0	0 mM $\text{Ca}^{2+}$ buffer
3	20 mM caffeine, 0 mM $\text{Ca}^{2+}$
6	0 mM $\text{Ca}^{2+}$
9	20 mM caffeine, 0 mM $\text{Ca}^{2+}$
12	0 mM $\text{Ca}^{2+}$
15	20 mM caffeine, 0 mM $\text{Ca}^{2+}$
18	2 mM $\text{Ca}^{2+}$
24	20 mM caffeine, 2 mM $\text{Ca}^{2+}$
27	2 mM $\text{Ca}^{2+}$
30	20 mM caffeine, 2 mM $\text{Ca}^{2+}$
33	2 mM $\text{Ca}^{2+}$
36	20 mM caffeine, 2 mM $\text{Ca}^{2+}$
39	2 mM $\text{Ca}^{2+}$
44	10 $\mu\text{M}$ ACh
49	2 mM $\text{Ca}^{2+}$ buffer wash
59	end point



**Figure 3.12 Extracellular  $\text{Ca}^{2+}$  is required for internal  $\text{Ca}^{2+}$  store release to induce airway smooth muscle contraction in mouse peripheral airways.** Airway tracing in a 130  $\mu\text{m}$  thick, C57BL/6 WT mouse lung slice (representative of  $n = \text{four mice}$ ). After perfusion with 0 mM  $\text{Ca}^{2+}$  buffer, an initial transient contraction was seen to 20 mM caffeine, no other contractions were seen with further perfusion of 20 mM caffeine in 0 mM  $\text{Ca}^{2+}$  buffer. The slice was then washed with 2 mM  $\text{Ca}^{2+}$ ,  $\text{Ca}^{2+}$  stores were replenished with intermittent perfusion of 2 mM  $\text{Ca}^{2+}$  buffer between transient contractions induced by 20 mM caffeine. A maximum contractile response maintained to 10  $\mu\text{M}$  ACh was shown prior to final wash out in 2 mM  $\text{Ca}^{2+}$ .





**Figure 3.13** Extracellular  $\text{Ca}^{2+}$  is required for internal  $\text{Ca}^{2+}$  store release to induce airway smooth muscle contraction in pig peripheral airways. Original airway tracing in a 170  $\mu\text{m}$  thick, pig lung slice (representative of  $n = \text{four airways, two pigs}$ ). After perfusion with 0 mM  $\text{Ca}^{2+}$  buffer, an initial transient contraction was seen to 20 mM caffeine, no other contractions were seen to further perfusion with 20 mM caffeine in 0 mM  $\text{Ca}^{2+}$  buffer. The slice was then washed with 2 mM  $\text{Ca}^{2+}$ ,  $\text{Ca}^{2+}$  stores were replenished with intermittent perfusion of 2 mM  $\text{Ca}^{2+}$  buffer between transient contractions induced by 20 mM caffeine. A maximum contractile response maintained to 1  $\mu\text{M}$  ACh was shown prior to final wash out in 2 mM  $\text{Ca}^{2+}$ .

In both mouse and pig, following the initial challenge with caffeine in 0 mM  $\text{Ca}^{2+}$  buffer a small contraction was induced. However, in the absence of extracellular  $\text{Ca}^{2+}$  further challenge with caffeine failed to induce additional contractions. When this experiment was repeated in the presence of extracellular  $\text{Ca}^{2+}$  at physiological levels, repeated contractions to caffeine could be induced.

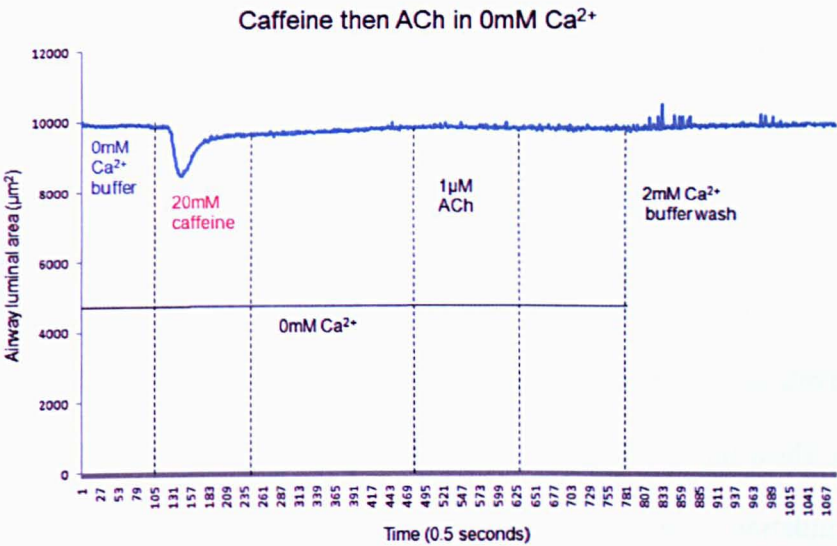
Having demonstrated that store replenish and release is required for ASM contraction (figure 3.12, 3.13); I next explored the roles of the sarcoplasmic reticulum (SR) located  $\text{IP}_3\text{R}$  and  $\text{RyR}$  in  $\text{Ca}^{2+}$  release for initiating contraction and regulating  $[\text{Ca}^{2+}]$  and also, whether separate  $\text{Ca}^{2+}$  sub-stores supplied these channels.



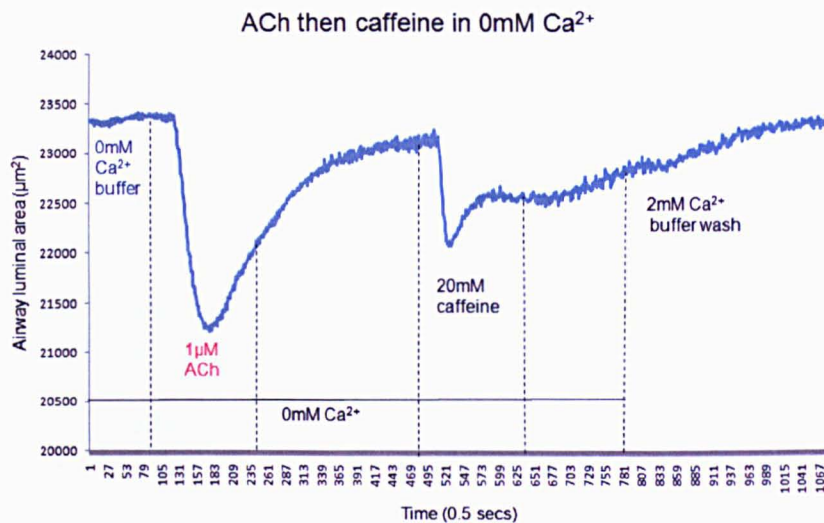
These studies were performed using pig lung slices only due to tissue availability at the time. Prior to experiments all lung slices were kept in 2 mM extracellular  $\text{Ca}^{2+}$ . The protocols involved initial perfusion with 0 mM  $\text{Ca}^{2+}$  (with 1 mM EGTA, a chelator preferential for  $\text{Ca}^{2+}$ ) any  $\text{Ca}^{2+}$  already in the internal stores was then released by activation of either the  $\text{IP}_3\text{R}$  (through stimulation with ACh) or RyR (through stimulation with 20 mM caffeine). The lung slice was further perfused with 0 mM  $\text{Ca}^{2+}$  followed by stimulation of the alternative SR located channel. The slice was then further perfused with 0 mM  $\text{Ca}^{2+}$ . Finally, 2 mM  $\text{Ca}^{2+}$  was reintroduced for ten minutes (to measure any contraction induced due to SOCC activation from prolonged store depletion of  $\text{Ca}^{2+}$  and sudden influx of reinstated  $\text{Ca}^{2+}$ ). The logic being that if the  $\text{IP}_3\text{R}$  is directly involved in contraction then after initial depletion of internal stores through the RyR pathway, ACh would not be able to induce further contraction (as shown in figure 3.14) (although this assumes a common  $\text{Ca}^{2+}$  store for  $\text{IP}_3\text{R}$  and RyR release). However, if the main role of the RyR is internal store regulation (rather than contraction) and therefore RyR has access to  $\text{Ca}^{2+}$  from separate sub-stores then a second contraction to stimulation of caffeine would be possible (as is shown figure 3.15).

Table 3.5 Perfusion system timings designed to highlight differences in  $\text{Ca}^{2+}$  store arrangement and receptor roles of the  $\text{IP}_3\text{R}$  and  $\text{RyR}$ ; required for airway smooth muscle contraction or internal  $\text{Ca}^{2+}$  store regulation. 1 mM EGTA was present in 0 mM  $\text{Ca}^{2+}$  buffer throughout the protocol (protocol described in text).

IP <sub>3</sub> and RyR effects on contraction		
Time (minutes)	Perfusion	Perfusion
0	0 mM $\text{Ca}^{2+}$ buffer	0 mM $\text{Ca}^{2+}$ buffer
3	1 $\mu\text{M}$ ACh, 0 mM $\text{Ca}^{2+}$	20 mM caffeine, 0 mM $\text{Ca}^{2+}$
8	0 mM $\text{Ca}^{2+}$	0 mM $\text{Ca}^{2+}$
16	20 mM caffeine, 0 mM $\text{Ca}^{2+}$	1 $\mu\text{M}$ ACh, 0 mM $\text{Ca}^{2+}$
21	0 mM $\text{Ca}^{2+}$	0 mM $\text{Ca}^{2+}$
26	2 mM $\text{Ca}^{2+}$ buffer wash	2 mM $\text{Ca}^{2+}$ buffer wash
36	end point	end point



**Figure 3.14** Following  $\text{RyR}$  stimulated contraction, no further contraction can be induced by ACh stimulation in pig peripheral airways. Representative tracing of pig peripheral airway lumen ( $n = \text{four airways, two pigs}$ ), in a  $170\ \mu\text{m}$  thick pig lung slice. The tracing shows initial perfusion of 0 mM  $\text{Ca}^{2+}$  buffer (with 1 mM EGTA) which was maintained throughout the majority of the experiment. A contraction was then stimulated by 20 mM caffeine ( $\text{RyR}$  release). The slice was further perfused with 0 mM  $\text{Ca}^{2+}$  buffer followed by 1  $\mu\text{M}$  ACh ( $\text{IP}_3\text{R}$   $\text{Ca}^{2+}$  release). 0 mM  $\text{Ca}^{2+}$  buffer was then perfused followed by 2 mM  $\text{Ca}^{2+}$  buffer. In the absence of extracellular  $\text{Ca}^{2+}$  an initial transient contraction was induced to caffeine via  $\text{RyR}$  release. Following this store depletion, no further contraction was seen with ACh stimulation of the  $\text{IP}_3\text{R}$ . Despite prolonged  $\text{Ca}^{2+}$  store deprivation, the reintroduction of extracellular  $\text{Ca}^{2+}$  did not induce a contraction.

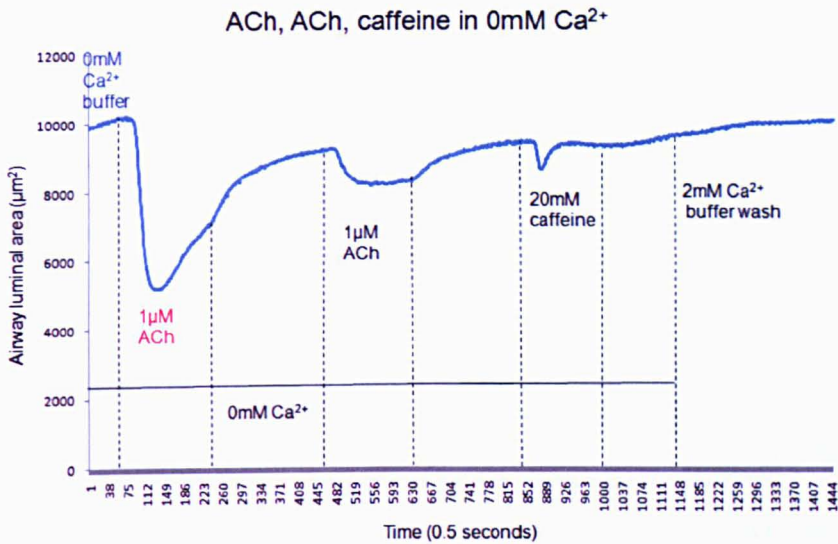


**Figure 3.15** Following  $IP_3R$  stimulated contraction, further contraction is seen to RyR induced stimulation in pig peripheral airways. Representative tracing of pig peripheral airway lumen ( $n =$  four airways, two pigs), in a  $170\ \mu\text{m}$  thick pig lung slice. The tracing shows initial perfusion of  $0\ \text{mM}\ \text{Ca}^{2+}$  buffer (with added EGTA) maintained throughout the majority of the experiment. A contraction was then stimulated by  $1\ \mu\text{M}$  ACh ( $\text{Ca}^{2+}$  release via the  $IP_3R$ ). After further perfusion of  $0\ \text{mM}\ \text{Ca}^{2+}$ , a smaller contraction was observed with stimulation from  $20\ \text{mM}$  caffeine (RyR release). The slice was then perfused with  $0\ \text{mM}$  and finally  $2\ \text{mM}\ \text{Ca}^{2+}$ . Despite initial store depletion via the  $IP_3R$ , the contraction observed with caffeine stimulation may suggest another existing supply of  $\text{Ca}^{2+}$  for the RyR. No contraction was induced with the reintroduction of extracellular  $\text{Ca}^{2+}$  at the end of the protocol.

These results suggest that  $IP_3R$  release is directly involved in the pathway to activate contraction and must share  $\text{Ca}^{2+}$  stores with the RyR (figure 3.14). Results also show that the RyR may have access to separate internal  $\text{Ca}^{2+}$  stores as after ACh stimulation, a second contraction is seen to caffeine (figure 3.15). This supports the notion that the RyR is involved in regulation of  $[\text{Ca}^{2+}]_i$  rather than contraction maintenance (and would be consistent with the transient contraction and variable magnitude of contraction often seen with caffeine stimulation). However, it is also possible that a single ACh stimulation does not release all  $\text{Ca}^{2+}$  from the store. Therefore, this protocol was extended to incorporate a second challenge to ACh prior to stimulation with caffeine (figure 3.16). A contraction was seen with a second

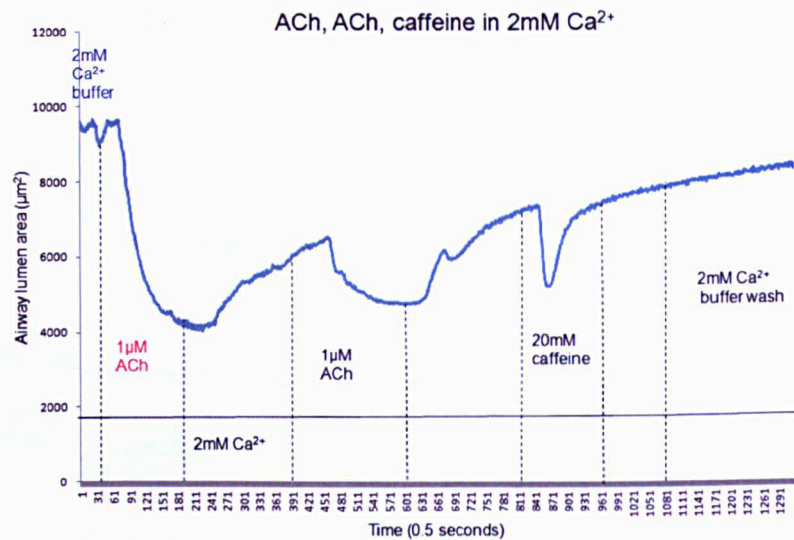


stimulation of ACh (although magnitude of contraction was reduced) and seen again with caffeine stimulation.



**Figure 3.16 Despite depletion of intracellular stores, repeated airway contractions are induced to IP<sub>3</sub>R and RyR release.** Representative tracing of pig peripheral airway lumen ( $n = \text{four airways, two pigs}$ ), in a  $170 \mu\text{m}$  thick pig lung slice. The tracing shows initial perfusion of  $0 \text{ mM Ca}^{2+}$  buffer (with added EGTA), maintained throughout the majority of the experiment. A contraction was then stimulated by  $1 \mu\text{M ACh}$  ( $\text{Ca}^{2+}$  release via the IP<sub>3</sub>R). After further perfusion of  $0 \text{ mM Ca}^{2+}$ , another much smaller contraction was seen to ACh, further  $0 \text{ mM Ca}^{2+}$  was perfused, followed by a final small contraction to  $20 \text{ mM caffeine}$  (RyR release). The slice was then perfused with  $0 \text{ mM}$  and finally  $2 \text{ mM Ca}^{2+}$ . Despite initial store depletion via the IP<sub>3</sub>R, a second contraction was observed through this pathway of  $\text{Ca}^{2+}$  release. A final contraction was observed with caffeine stimulation of the RyR. No contraction was induced with the reintroduction of extracellular  $\text{Ca}^{2+}$ .

Following the same protocol as in figure 3.16, a control experiment with  $2 \text{ mM}$  extracellular  $\text{Ca}^{2+}$  was included to demonstrate that magnitude of contraction is reliant on  $\text{Ca}^{2+}$  supply. Results show that stimulation of IP<sub>3</sub>R and RyR release produces repeated significant contractions (figure 3.17); as compared with figure 3.16 showing a reduced contraction after  $0 \text{ mM Ca}^{2+}$ .



**Figure 3.17 Magnitude of contraction is reliant on  $\text{Ca}^{2+}$  supply.** Representative tracing of pig peripheral airway lumen ( $n = \text{four airways, two pigs}$ ), in a  $170 \mu\text{m}$  thick pig lung slice. The tracing shows initial perfusion of  $2 \text{ mM } \text{Ca}^{2+}$  buffer, maintained throughout the majority of the experiment. A contraction was then stimulated by  $1 \mu\text{M ACh}$  ( $\text{Ca}^{2+}$  release via the  $\text{IP}_3\text{R}$ ). After further perfusion of  $2 \text{ mM } \text{Ca}^{2+}$ , another contraction was seen to ACh, further  $2 \text{ mM } \text{Ca}^{2+}$  was perfused, followed by a final contraction to  $20 \text{ mM caffeine}$  ( $\text{RyR}$  release). The slice was then perfused with  $2 \text{ mM } \text{Ca}^{2+}$ . Repeated contractions with large magnitudes were observed via stimulation of the  $\text{IP}_3\text{R}$  and with caffeine stimulation of the  $\text{RyR}$ .

These results suggest that a  $\text{Ca}^{2+}$  store reserve may supply the  $\text{RyR}$  release pathway only which could possibly mobilise  $\text{RyR}$  stored  $\text{Ca}^{2+}$  to supply the  $\text{IP}_3\text{R}/\text{RyR}$  shared pathway. Alternatively, this may reflect an inability by ACh to deplete the stores completely. The roles of the  $\text{IP}_3\text{R}$  and  $\text{RyR}$  are explored further in the next section using prolonged SERCA pump inhibition (figure 3.19, figure 3.20).

### 3.3.4 Influence of SERCA pump inhibitors on magnitude and maintenance of contraction

The following experiments were designed to study the influence of inhibition of  $\text{Ca}^{2+}$  uptake into internal stores (and therefore supply of  $\text{Ca}^{2+}$  to be released for

cytosolic  $\text{Ca}^{2+}$  increase) on the ability to induce and maintain a contraction. Parallel experiments were run using both SERCA pump inhibitors, cyclopiazonic acid (CPA) and thapsigargin. As both inhibitors produced similar results, only CPA data is shown here. Perfusion system timings were designed to show an initial control contraction to ACh. Throughout the protocol, airways were perfused with varying conditions: CPA and ACh together for five minutes and both CPA and ACh separately. An eight minute interval for replenish of internal  $\text{Ca}^{2+}$  stores was included. Combinations were designed to determine whether perfusion with CPA (prior to/ or in combination with) perfusion with ACh affected the magnitude of contraction (see table 3.6). Results are shown for both mouse and pig with representative airway tracings and collective data (figures: 3.17, 3.18).

*Table 3.6 Perfusion system timings designed to show effect of SERCA pump inhibition using CPA, on magnitude and maintenance of ACh induced contraction.*

**Effects of CPA on ACh induced contraction**

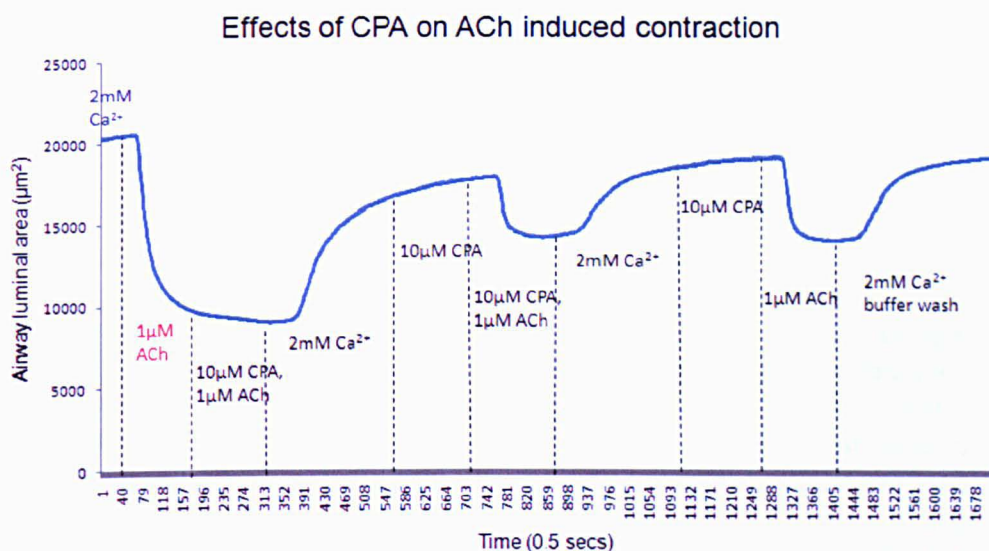
Time (minutes)	Perfusion
0	2 mM $\text{Ca}^{2+}$ buffer
1	1 $\mu\text{M}$ ACh
6	10 $\mu\text{M}$ CPA, 1 $\mu\text{M}$ ACh
11	2 mM $\text{Ca}^{2+}$
19	10 $\mu\text{M}$ CPA
24	10 $\mu\text{M}$ CPA, 1 $\mu\text{M}$ ACh
29	2 mM $\text{Ca}^{2+}$
37	10 $\mu\text{M}$ CPA
42	1 $\mu\text{M}$ ACh
47	2 mM $\text{Ca}^{2+}$ buffer
	wash
57	end point



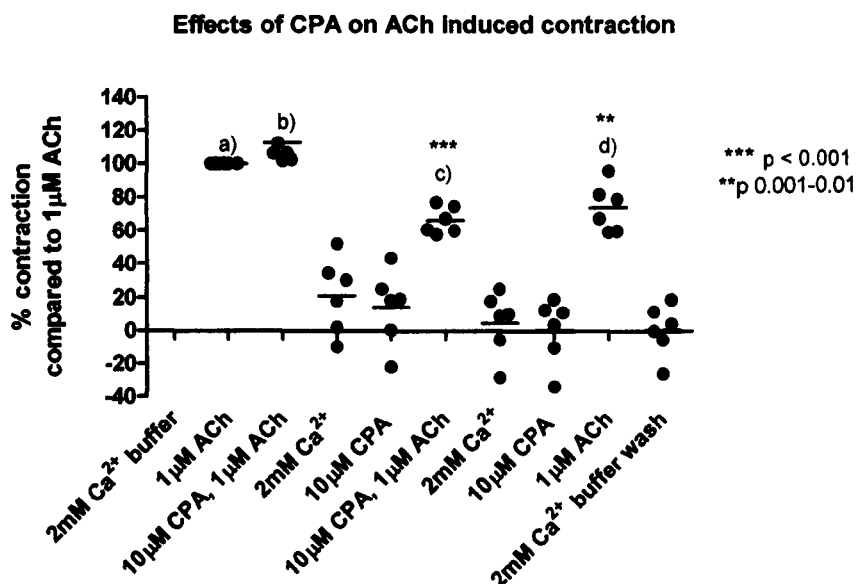




with pre-treatment with CPA alone. Data for b) 10  $\mu\text{M}$  CPA, 10  $\mu\text{M}$  ACh, c) 10  $\mu\text{M}$  CPA, 10  $\mu\text{M}$  ACh and d) final 10  $\mu\text{M}$  ACh were analysed using one-way ANOVA followed by Dunnett's post test comparing all means to the control; a) initial 10  $\mu\text{M}$  ACh. Results were not significant with all  $p$  values  $> 0.05$ . Note: the airways had not fully relaxed to baseline levels with the second perfusion of 2 mM  $\text{Ca}^{2+}$  buffer alone as indicated at e), baseline drift is discussed in section 3.4.



**Figure 3.18a SERCA pump inhibition significantly reduces magnitude of contraction in pig peripheral airways.** Example of an airway tracing representing contractile responses in  $n =$  six airways, three pig lung slices. The slice was perfused with 2 mM  $\text{Ca}^{2+}$  buffer, a contraction was shown to ACh followed immediately by perfusion with 10  $\mu\text{M}$  CPA and 1  $\mu\text{M}$  ACh. The slice was then washed with buffer, pretreated with 10  $\mu\text{M}$  CPA and then perfused with both CPA and ACh. Finally, the slice was washed again with buffer, pretreated with 10  $\mu\text{M}$  CPA and then perfused with ACh (1  $\mu\text{M}$ ) alone. Final wash out to buffer was shown. A significant reduction in contraction to ACh (alone and in presence of CPA) was shown after perfusion with CPA alone for five minutes.



**Figure 3.18b SERCA pump inhibition significantly reduces magnitude of contraction in pig peripheral airways.** Results for  $n =$  six airways, three pigs, normalised to 2 mM  $\text{Ca}^{2+}$  buffer alone as 0% contraction and 1  $\mu\text{M}$  ACh as 100% contractile response. 2 mM  $\text{Ca}^{2+}$  buffer was maintained throughout the experiment. Individual dots represent one airway, blue lines indicate the mean value for that group. Data for b) ACh and CPA with no pre-treatment with CPA, c) ACh and CPA with pre-treatment with CPA alone and d) final ACh alone with pre-treatment with CPA alone were analysed using one-way ANOVA followed by Dunnett's post test comparing all means to the control; a) initial 10  $\mu\text{M}$  ACh. Contractions of similar magnitude are shown for a) ACh alone (100% contraction) and b) ACh and CPA (mean = 112.3% airway contraction). However, pre-treatment with CPA alone (five minute perfusion) followed by c) ACh and CPA significantly reduced the magnitude of contraction ( $p < 0.001$ , mean = 66.3% contraction), as did pre-treatment with CPA alone followed by d) ACh alone ( $p = 0.001 - 0.01$ , mean = 73.7 % airway contraction).

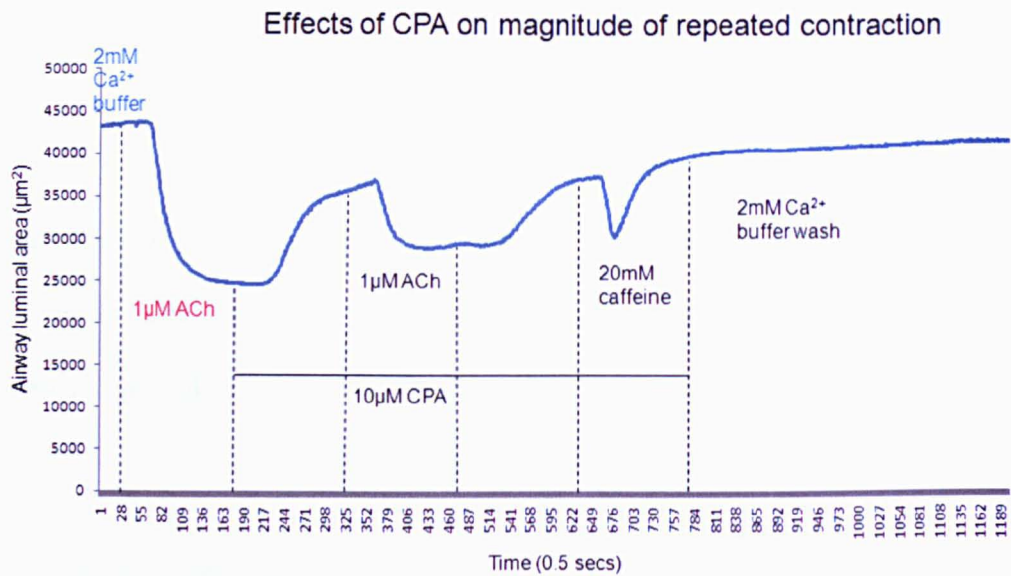
These results indicate differences between mouse and pig in CPA sensitivity or SR store release regulation for contraction. In pig magnitude of contraction appears to correlate with the amount of stored  $\text{Ca}^{2+}$  available for release.

Results in section 3.3.3 hinted at differing roles and  $\text{Ca}^{2+}$  store supplies for the  $\text{IP}_3\text{R}$  and  $\text{RyR}$ . To further investigate the roles of these channels and their relationship to magnitude of contraction by preventing internal store uptake using a SERCA pump inhibitor, CPA; 2 mM extracellular  $\text{Ca}^{2+}$  was maintained throughout

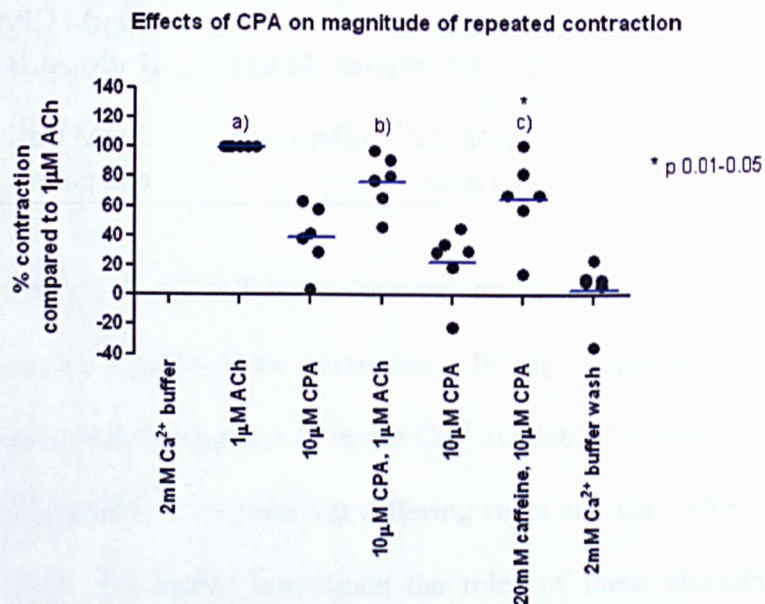
the experiment. This was studied by using an initial maximum contraction to either ACh or caffeine followed by perfusion with CPA alone (CPA was then present until wash out), followed by stimulation with ACh or caffeine by the same release pathway. After further CPA perfusion, a final contraction was stimulated by the alternative release channel (to determine if contraction would be absent/significantly reduced in magnitude). Due to tissue availability, the following experiments were performed for pig lung slices only (figures: 3.19, 3.20).

*Table 3.7 Perfusion system timings using SERCA pump inhibition (by CPA) to highlight differing roles of the RyR (caffeine stimulation) and IP<sub>3</sub>R (ACh stimulation) release pathways in influencing magnitude of contraction.*

<b>Effects of CPA on magnitude of repeated contraction</b>		
<b>Time (minutes)</b>	<b>Perfusion</b>	<b>Perfusion</b>
0	2 mM Ca <sup>2+</sup> buffer	2 mM Ca <sup>2+</sup> buffer
1	1 $\mu$ M ACh	20 mM caffeine
6	10 $\mu$ M CPA	10 $\mu$ M CPA
14	1 $\mu$ M ACh, 10 $\mu$ M CPA	20 mM caffeine, 10 $\mu$ M CPA
19	10 $\mu$ M CPA	10 $\mu$ M CPA
27	20 mM caffeine, 10 $\mu$ M CPA	1 $\mu$ M ACh, 10 $\mu$ M CPA
32	2 mM Ca <sup>2+</sup> buffer wash	2 mM Ca <sup>2+</sup> buffer wash
42	end point	end point



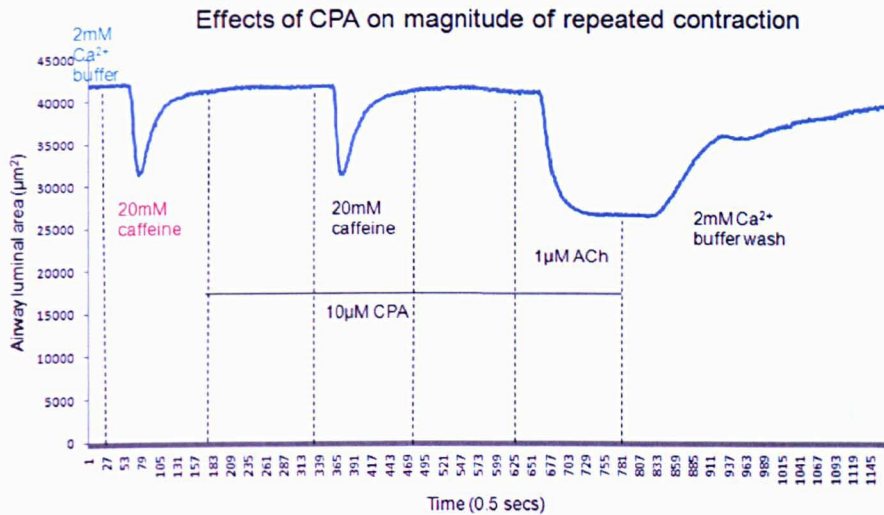
**Figure 3.19a SERCA pump inhibition reduces magnitude of contraction following  $IP_3R$  stimulation in pig peripheral airways.** Example of an airway tracing representing contractile responses in  $n =$  six airways, three pig lung slices. The slice was perfused with 2 mM  $Ca^{2+}$  buffer, a contraction was shown to ACh followed by perfusion with 10  $\mu M$  CPA, then CPA with 1  $\mu M$  ACh. Further CPA was perfused followed by 20 mM caffeine with CPA. Finally the slice was washed with 2 mM  $Ca^{2+}$ . In this experiment, following  $IP_3R$  stimulation, the magnitude of further contractions via  $IP_3R$  release and RyR release appeared to be reduced in the presence of CPA. Note: the airways did not relax back to base line in between CPA washes.



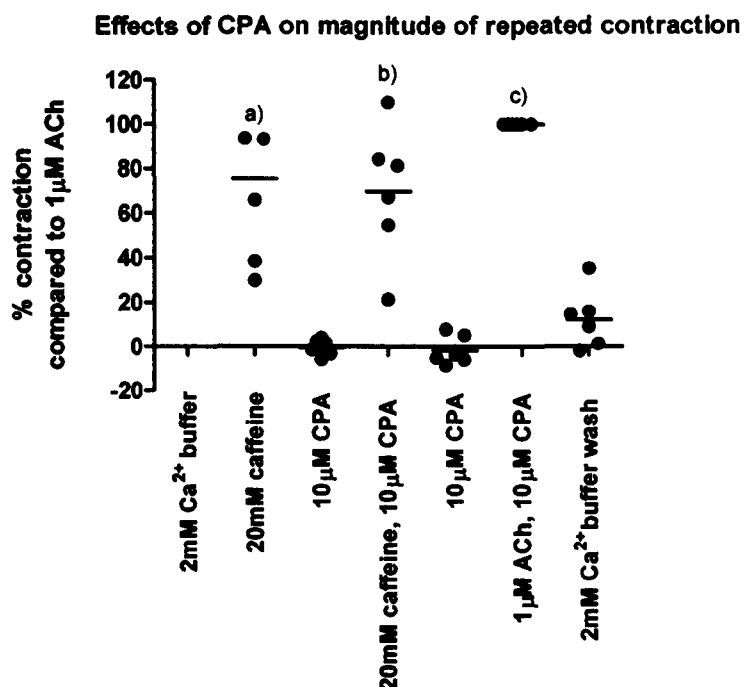
**Figure 3.19b SERCA pump inhibition reduces magnitude of contraction through RyR release following  $IP_3R$  stimulation in pig peripheral airways.** Results for  $n =$  six airways, three pigs, normalised to 2 mM  $Ca^{2+}$  buffer alone as 0% contraction and 1  $\mu M$  ACh as 100% contractile response. 2 mM  $Ca^{2+}$  buffer was maintained throughout the experiment. Individual dots represent one airway, blue lines indicate



the mean value for that group. Data were analysed using one-way ANOVA followed by Dunnett's post test comparing all means to the control; a) initial  $1\ \mu\text{M}$  ACh. Data for b)  $10\ \mu\text{M}$  CPA,  $10\ \mu\text{M}$  ACh, showed a reduction in magnitude of contraction via  $\text{IP}_3\text{R}$  release, however this was not significant (mean = 76.4%). Final stimulation via the RyR also produced a contraction of decreased magnitude ( $p = 0.01 - 0.05$ , mean = 65.2%). During CPA washes, the airways did not relax to base line levels.



**Figure 3.20a** Following RyR release,  $\text{IP}_3\text{R}$  stimulation does not lead to reduction in magnitude of contraction despite prolonged SERCA pump inhibition. Example of an airway tracing representing contractile responses in  $n = \text{six}$  airways, three pig lung slices. The slice was perfused with  $2\ \text{mM}$   $\text{Ca}^{2+}$  buffer, a contraction was shown to caffeine followed immediately by perfusion with  $10\ \mu\text{M}$  CPA, then CPA with caffeine. Again CPA was perfused followed by a final contraction to ACh. Finally the slice was washed with  $2\ \text{mM}$   $\text{Ca}^{2+}$ . In this experiment, following initial contraction to RyR release, the magnitude of the second contraction stimulated through RyR release was not reduced in magnitude, nor was the final contraction via ACh stimulated  $\text{IP}_3\text{R}$  release.



**Figure 3.20b** Following RyR release, IP<sub>3</sub>R stimulation does not lead to reduction in magnitude of contraction despite prolonged SERCA pump inhibition. Results for  $n =$  six airways, three pigs, normalised to 2 mM Ca<sup>2+</sup> buffer alone as 0% contraction and 1 μM ACh as 100% contractile response. 2 mM Ca<sup>2+</sup> buffer was maintained throughout the experiment. Individual dots represent one airway, blue lines indicate the mean value for that group. Data were analysed using one-way ANOVA followed by Dunnett's post test comparing all means to the control; a) initial 20 mM caffeine (mean = 75.6%). A second contraction was shown to caffeine b) (mean = 69.7%). Although there was a trend towards a higher magnitude of contraction being produced to ACh (c), results are not significantly different.

With SERCA pump inhibition, these results showed a reduction in magnitude of contraction following repeated stimulation of the IP<sub>3</sub>R pathway (3.19). However no reduction in degree of contraction was shown for IP<sub>3</sub>R release after repeated RyR stimulation (3.20). Results from sections 3.3.3 and 3.3.4 suggest that i) IP<sub>3</sub>R release is directly involved in contraction and shares a common store supply with RyR. ii) SERCA pump inhibition reduces the magnitude of contraction in pig peripheral airways. iii) Importantly, results suggest differences between mouse and pig in internal Ca<sup>2+</sup> store regulation required for release for contraction.



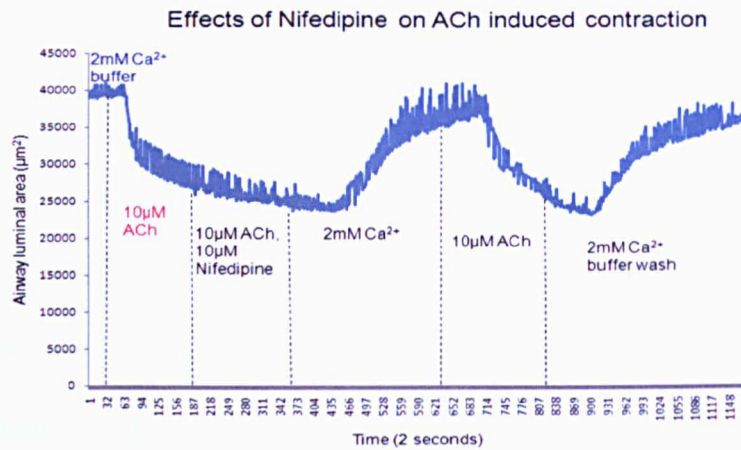
### 3.3.5 Significance of Voltage Operated Calcium Channels (VOCC) for $\text{Ca}^{2+}$ entry to maintain contraction

In the next set of experiments I explored the contribution of VOCCs to  $\text{Ca}^{2+}$  entry for maintaining ASM contraction using the L-type VOCC inhibitor, nifedipine. As can be seen in figure 3.21 and 3.22, nifedipine had no relaxant effect on ACh induced contractions. The protocol involved an initial contraction to ACh followed by the addition of nifedipine with ACh present, to compare magnitude (table 3.8). The corresponding representative airway contraction tracing and mean values are shown for mouse and pig (figures: 3.21, 3.22).

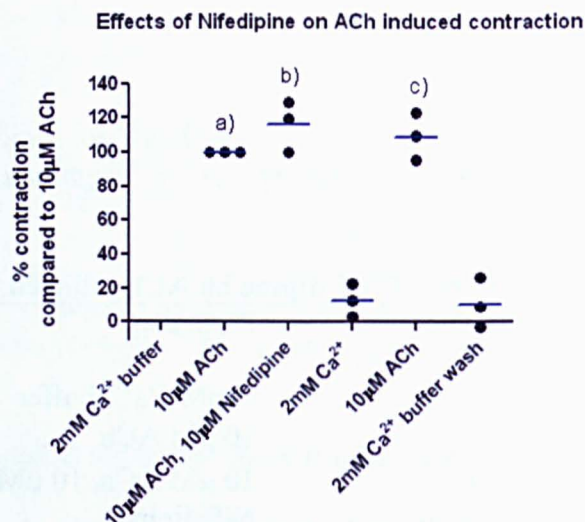
*Table 3.8 Perfusion system timings designed to show effect of voltage operated calcium channel inhibition (nifedipine) on magnitude and maintenance of ACh induced contraction.*

#### **Effects of Nifedipine on ACh induced contraction**

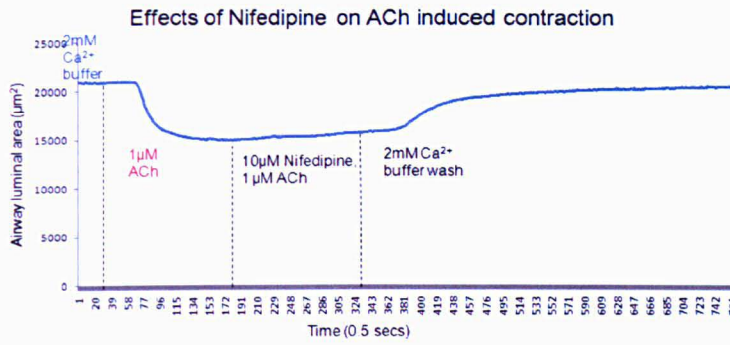
Time (minutes)	Perfusion
0	2 mM $\text{Ca}^{2+}$ buffer
1	10 $\mu\text{M}$ ACh
6	10 $\mu\text{M}$ ACh, 10 $\mu\text{M}$ Nifedipine
11	2 mM $\text{Ca}^{2+}$
19	10 $\mu\text{M}$ ACh
24	2 mM $\text{Ca}^{2+}$ buffer wash



**Figure 3.21a Nifedipine does not induce airway relaxation in mouse peripheral airways.** Representative airway tracing for a C57BL/6 lung slice (representative of  $n = \text{three}$ , three mice) showing initial buffer perfusion, pre-contraction with  $10 \mu\text{M}$  ACh, perfusion of  $10 \mu\text{M}$  nifedipine with ACh, wash, contraction to  $10 \mu\text{M}$  ACh and final wash out to relaxed state.

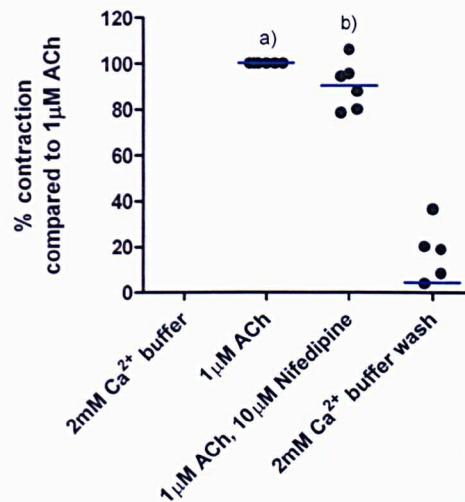


**Figure 3.21b Nifedipine does not induce airway relaxation in mouse peripheral airways.** Results for percentage contractile response in ( $n = \text{three}$ , three mice)  $130 \mu\text{m}$  thick, C57BL/6 mouse lung slices. Individual dots indicate one airway from one mouse, blue lines indicate mean values. Means for pre-contraction with a) initial  $10 \mu\text{M}$  ACh, perfusion, b)  $10 \mu\text{M}$  nifedipine with ACh and final contraction to c)  $10 \mu\text{M}$  ACh are shown. Results indicate that nifedipine does not significantly contribute to airway relaxation. Analysed using one-way AVOVA and Dunnett's post test comparing means for b) and c) to a)  $10 \mu\text{M}$  ACh, all results were not significant,  $p$  values  $> 0.05$ .



**Figure 3.22a** *Nifedipine does not induce airway relaxation in pig peripheral airways.* Representative airway tracing in a pig lung slice (representative of  $n =$  six, three pigs) showing initial buffer perfusion, pre-contraction with  $1 \mu\text{M}$  ACh, perfusion of  $10 \mu\text{M}$  nifedipine with ACh and final wash out to relaxed state.

**Effects of Nifedipine on ACh induced contraction**



**Figure 3.22b** *Nifedipine does not induce airway relaxation in pig peripheral airways.* Results for percentage contractile response in ( $n =$  six airways, three pigs) pig lung slices. Individual dots indicate one airway, blue lines indicate mean values. Means for pre-contraction with a)  $1 \mu\text{M}$  ACh perfusion were compared with b)  $10 \mu\text{M}$  nifedipine with ACh (mean =  $90.3\%$  contraction) using one-way ANOVA and Dunnett's post test.

### 3.3.6 Characterisation of inhibitory response to selected calcium channel binding agents and Store Operated Calcium Channel (SOCC) inhibitors

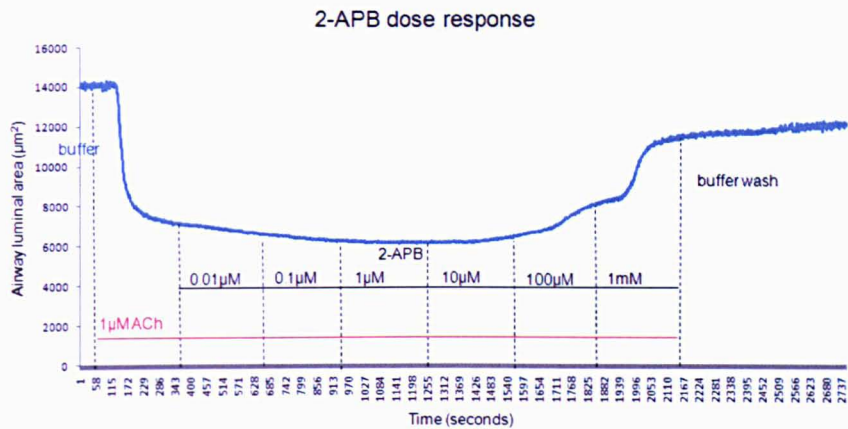
Given the results suggesting no apparent role for VOCCs in control of peripheral airway contraction/ relaxation in both mouse and pig, I next explored the contribution of store operated calcium channel entry (SOCC) for maintained airway contraction. These experiments were complicated by a lack of specific SOCC

inhibitors. SKF-96365, a commonly used SOCC inhibitor in other cell types, does not inhibit SOCC  $\text{Ca}^{2+}$  influx in smooth muscle; instead showing inhibition of ROCC and other non specific effects (Guibert, Marthan et al. 2004). Both  $\text{La}^{3+}$  and  $\text{Ni}^{2+}$  inhibit SOCC in a variety of smooth muscle types, however, they also inhibit ROCC in others (McFadzean and Gibson 2002). 2-APB is shown to inhibit  $\text{IP}_3$  receptor mediated  $\text{Ca}^{2+}$  release at concentrations of 1 - 10  $\mu\text{M}$ . However, it also inhibits SOCC at concentrations > 100  $\mu\text{M}$  (Mizuta, Gallos et al. 2008). In all protocols, the airways were pre-contracted with 1  $\mu\text{M}$  ACh (sub maximal concentration) which was present throughout the dose response. All buffers, agonists and inhibitors were made up with 2 mM extracellular  $\text{Ca}^{2+}$ . For each inhibitor representative airway contraction tracings and corresponding graphed data for mouse (three airways from three mice) and pig (six airways from three pigs) are shown (figures: 3.23, 3.24, 3.25, 3.26, 3.27, 3.28, 3.29).

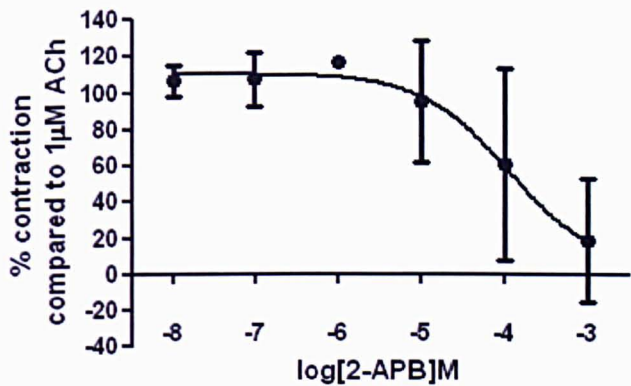
*Table 3.9 Perfusion system timings for store operated calcium channel inhibitor dose responses in the presence of ACh (described in text).*

<b>Inhibitory dose response for SOCCs (in 2 mM <math>\text{Ca}^{2+}</math>)</b>					
Time (minutes)	Nickel ( $\text{Ni}^{2+}$ )	2-APB	Lanthanum ( $\text{La}^{3+}$ )	JS (Icrac inhibitor)	N117 (Icrac inhibitor)
0	2 mM $\text{Ca}^{2+}$ buffer	2 mM $\text{Ca}^{2+}$ buffer	2 mM $\text{Ca}^{2+}$ buffer	2 mM $\text{Ca}^{2+}$ buffer	2 mM $\text{Ca}^{2+}$ buffer
1	1 $\mu\text{M}$ ACh (to precontract)	1 $\mu\text{M}$ ACh (to precontract)	1 $\mu\text{M}$ ACh (to precontract)	10 $\mu\text{M}$ ACh (to precontract)	10 $\mu\text{M}$ ACh (to precontract)
6	0.01 $\mu\text{M}$	0.01 $\mu\text{M}$	0.01 $\mu\text{M}$	0.001 $\mu\text{M}$	0.001 $\mu\text{M}$
11	0.1 $\mu\text{M}$	0.1 $\mu\text{M}$	0.1 $\mu\text{M}$	0.01 $\mu\text{M}$	0.01 $\mu\text{M}$
16	1 $\mu\text{M}$	1 $\mu\text{M}$	1 $\mu\text{M}$	0.1 $\mu\text{M}$	0.1 $\mu\text{M}$
21	10 $\mu\text{M}$	10 $\mu\text{M}$	10 $\mu\text{M}$	0.5 $\mu\text{M}$	0.5 $\mu\text{M}$
26	100 $\mu\text{M}$	100 $\mu\text{M}$	100 $\mu\text{M}$	1 $\mu\text{M}$	1 $\mu\text{M}$
31	1 mM	1 mM	1 mM	10 $\mu\text{M}$	10 $\mu\text{M}$
36	2 mM $\text{Ca}^{2+}$ buffer wash	2 mM $\text{Ca}^{2+}$ buffer wash	2 mM $\text{Ca}^{2+}$ buffer wash	2 mM $\text{Ca}^{2+}$ buffer wash	2 mM $\text{Ca}^{2+}$ buffer wash
46	end point	end point	end point	end point	end point

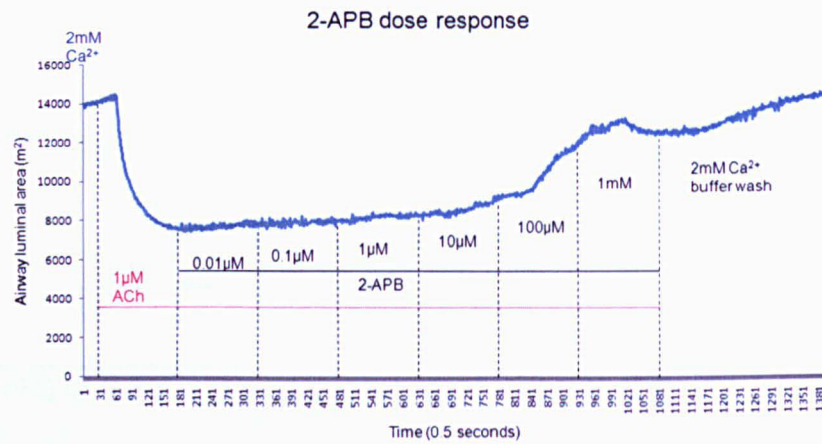




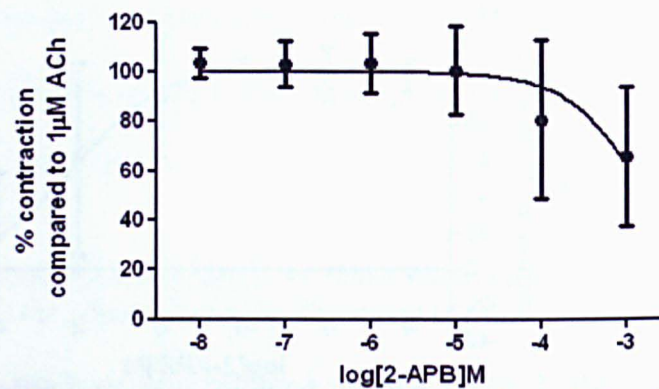
**Figure 3.23a** 2-APB produces airway relaxation in mouse peripheral airways. Representative airway tracing in a C57BL/6 WT mouse lung slice (representative of  $n = \text{three}$ , three mice). The slices were perfused with buffer then pre-contracted with 1  $\mu\text{M}$  ACh, which was present with the addition of ten-fold increasing concentrations of 2-APB from 0.01  $\mu\text{M}$  to 1 mM.



**Figure 3.23b** 2-APB produces airway relaxation in mouse peripheral airways. Results are shown for  $n = \text{three}$  airways from C57BL/6 WT mouse lung slices, normalised to buffer as 0% contraction and 1  $\mu\text{M}$  ACh as 100% contractile response. Data points represent mean values; error bars indicate SD. At higher concentrations ( $> 10 \mu\text{M}$ ), relaxation was seen. At 1 mM 2-APB,  $81.0 \pm 19.7\%$  airway relaxation was induced.

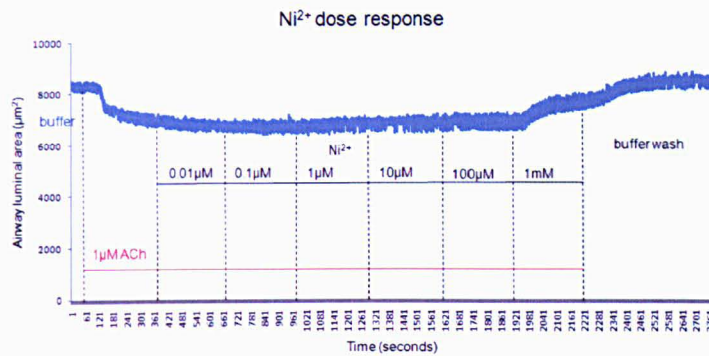


**Figure 3.24a 2-APB produces airway relaxation in pig peripheral airways.** Representative airway tracing in a pig lung slice (representative of  $n =$  six airways, three pigs). The slices were perfused with buffer then pre-contracted with  $1 \mu\text{M}$  ACh, which was present with the addition of ten-fold increasing concentrations of 2-APB from  $0.01 \mu\text{M}$  to  $1 \text{ mM}$ .



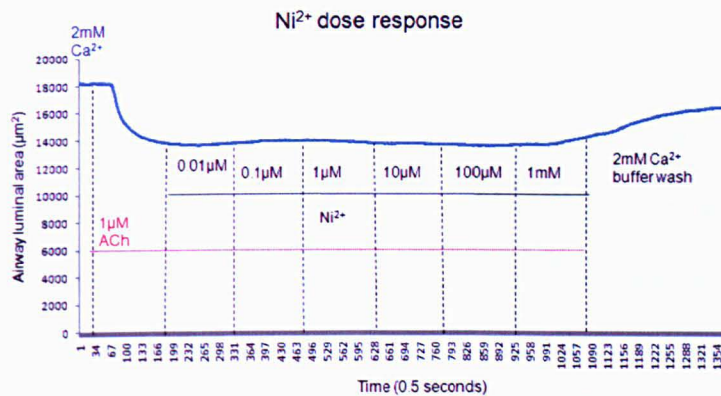
**Figure 3.24b 2-APB produces airway relaxation in pig peripheral airways.** Results are shown for  $n =$  six airways, three pigs. Normalised to buffer as 0% contraction and  $1 \mu\text{M}$  ACh as 100% contractile response. Data points represent mean values at each concentration. Error bars indicate SD. At  $1 \text{ mM}$  2-APB,  $34.4 \pm 12.6\%$  airway relaxation was measured.





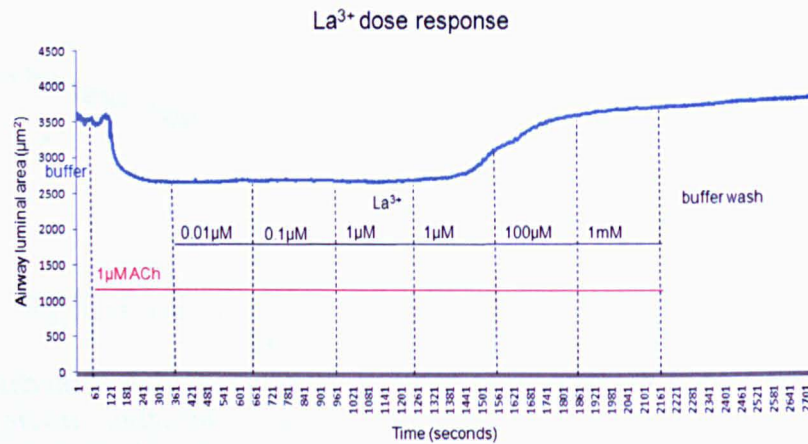
**Figure 3.25  $\text{Ni}^{2+}$  induces airway relaxation at high concentration in mouse peripheral airways.** Representative airway contraction tracing during a dose response to  $\text{Ni}^{2+}$  in a C57BL/6 WT mouse lung slice (representative of  $n = \text{three}$ , three mice). The slices were perfused with buffer then pre-contracted with  $1 \mu\text{M}$  ACh which was present throughout the dose response with the addition of ten-fold increasing concentrations of  $\text{Ni}^{2+}$  from  $0.01 \mu\text{M}$  to  $1 \text{mM}$ .

As the airways did not relax until a maximum contraction, mean values are not shown. At  $100 \mu\text{M}$   $\text{Ni}^{2+}$   $5.5 \pm 6.5\%$  relaxation was found. At maximum concentration ( $1 \text{mM}$ )  $36.2 \pm 3.5\%$  relaxation was measured ( $n = \text{three}$  airways from three mice).

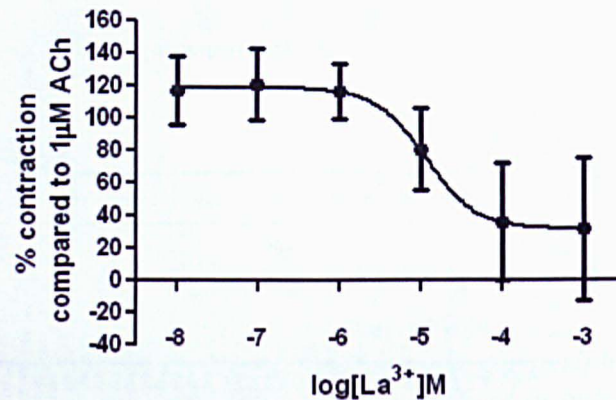


**Figure 3.26  $\text{Ni}^{2+}$  induces small airway relaxation at high concentration in pig peripheral airways.** Representative airway contraction tracing during a dose response to  $\text{Ni}^{2+}$  in a pig lung slice (representative of  $n = \text{six}$  airways, three pigs). The slices were perfused with buffer then pre-contracted with  $1 \mu\text{M}$  ACh which was present throughout the dose response with the addition of ten-fold increasing concentrations of  $\text{Ni}^{2+}$  from  $0.01 \mu\text{M}$  to  $1 \text{mM}$ .

Pig airways did not show relaxation until a maximum contraction, therefore mean values are not shown. At maximum concentration ( $1 \text{mM}$ ),  $12.0 \pm 8.6\%$  relaxation was measured.

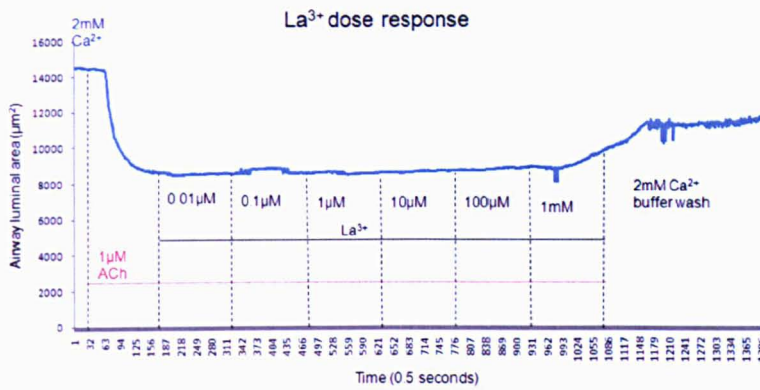


**Figure 3.27a**  $\text{La}^{3+}$  induces airway relaxation in mouse peripheral airways. A representative airway tracing for a dose response to  $\text{La}^{3+}$  in a C57BL/6 WT mouse lung slice (representative of  $n = \text{three}$ , three mice). The airway was pre-contracted with  $1 \mu\text{M}$  ACh, which was present with the addition of ten-fold increasing concentrations of  $\text{La}^{3+}$  from  $0.01 \mu\text{M}$  to  $1 \text{mM}$ . Relaxation was observed with  $\text{La}^{3+}$  concentrations above  $1 \mu\text{M}$ .



**Figure 3.27b**  $\text{La}^{3+}$  induces airway relaxation in mouse peripheral airways. Results for dose responses to  $\text{La}^{3+}$  in  $n = \text{three}$ , C57BL/6 WT mouse lung slices, normalised to buffer as 0% contraction and  $1 \mu\text{M}$  ACh as 100% contractile response. Data points represent mean values. Error bars indicate SD. An  $\text{IC}_{50}$  of  $12.1 \mu\text{M}$  and CI of  $2.8 \mu\text{M} - 50.9 \mu\text{M}$  was found. At  $1 \text{mM}$   $\text{La}^{3+}$ ,  $68.1 \pm 25.5\%$  relaxation was measured.





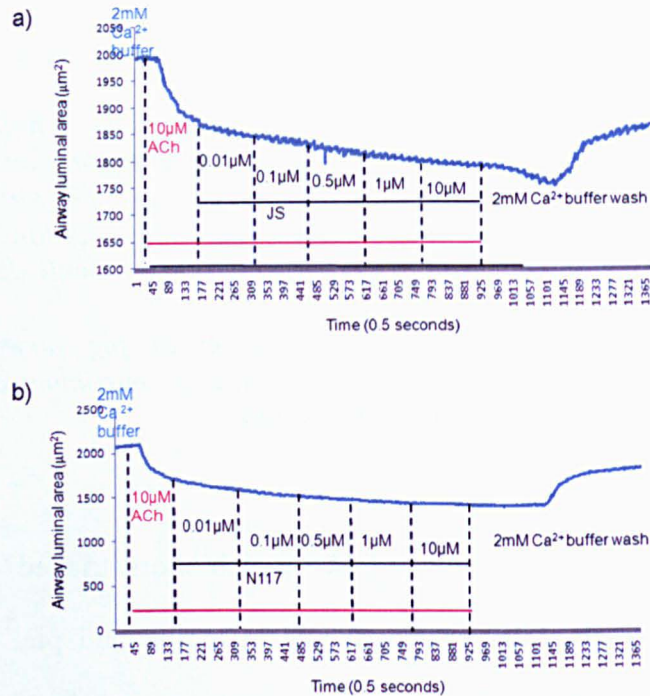
**Figure 3.28a**  $\text{La}^{3+}$  induces small airway relaxation at high concentration in pig peripheral airways. A representative airway tracing for a dose response to  $\text{La}^{3+}$  in a pig lung slice (representative of  $n = \text{six}$ , three pigs). The airway was pre-contracted with  $1 \mu\text{M}$  ACh, which was present with the addition of ten-fold increasing concentrations of  $\text{La}^{3+}$  from  $0.01 \mu\text{M}$  to  $1 \text{ mM}$ . A small degree of relaxation was observed at maximum concentration ( $1 \text{ mM}$ ).

As  $\text{La}^{3+}$  failed to induce relaxation of pig airways until a maximum concentration, mean values are not shown. At maximum concentration of  $1 \text{ mM}$   $\text{La}^{3+}$ ,  $5.2 \pm 8.9\%$  relaxation was measured.

As many of the reported SOCC inhibitors trialled did not induce airway relaxation in physiological conditions in mouse and pig; it was apparent that I needed a specific Icrac inhibitor and that airways had to be exposed to conditions that would favour SOCC in order to assess the potential contribution of SOCC. Icrac inhibitors were made available to me from GlaxoSmithKline: JS (GSK1349571A) and N117 (N11764-14-1)). The Icrac inhibitors are predominantly used for inhibition of T-cell and mast cell function ( $\text{IC}_{50}$  of  $100 \text{ nM} - 1 \mu\text{M}$ ). Interestingly, Icrac inhibition abolished OVA-evoked contractions of the rat trachea and allergen evoked contractions of the human bronchus (possibly through mast cell degranulation) (GSK data presented at Winter BPS, 2009).

Initially I followed the same protocol as for previous inhibitory dose response experiments; by first precontracting the airways with ACh which was present with increasing concentrations of the Icrac inhibitors. Representative airway tracings and

results are shown for both JS and N117 (figure 3.29). Experiments were performed in 2 mM  $\text{Ca}^{2+}$ ; to assess whether in physiological conditions, the Icrac inhibitors would prevent contraction maintenance.



**Figure 3.29 Icrac inhibitors (JS and N117) do not contribute to airway relaxation in pig peripheral airways under normal physiological conditions.** A representative airway tracing for a dose response to JS ( $n =$  seven, two pigs) and N117 ( $n =$  six, two pigs). The airways were pre-contracted with 10  $\mu\text{M}$  ACh, which was present with the addition of increasing concentrations of either JS (a) or N117 (b). Even at maximum concentration no relaxation was seen to the Icrac inhibitors in the presence of ACh and 2 mM  $\text{Ca}^{2+}$ .

Results suggest that in physiological conditions the Icrac inhibitors, JS (GSK1349571A) and N117 (N11764-14-1) did not contribute to airway relaxation in the presence of ACh (therefore mean results are not shown). Experiments were also performed to show an ACh dose response in lung slices that were either untreated, or pretreated with 10  $\mu\text{M}$  JS (GSK1349571A) and N117 (N11764-14-1) (maximum concentration) for an hour. The Icrac inhibitors were maintained throughout the

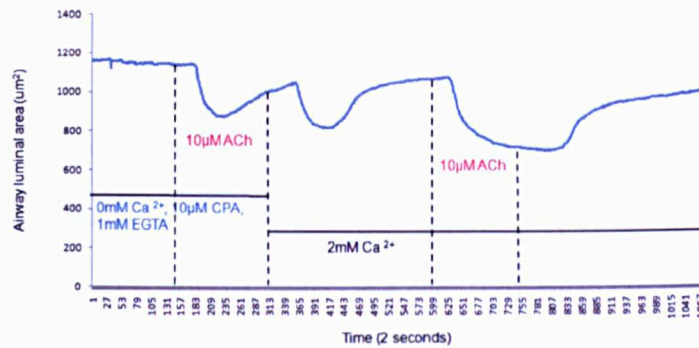
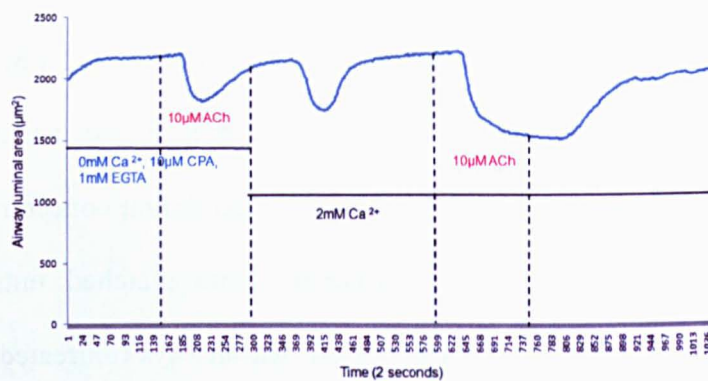
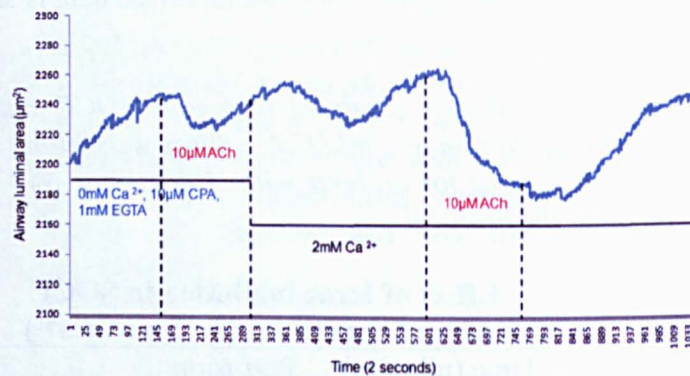
experiment. Again results suggested that Icrac inhibition did not prevent airway contraction maintenance (data not shown).

Therefore the following protocols were designed to investigate effects on contraction under conditions favouring SOCC activity. The protocol involved prolonged exposure to 0 mM  $\text{Ca}^{2+}$  (with EGTA, a chelator of  $\text{Ca}^{2+}$  and the SERCA pump inhibitor, CPA) followed by depletion of internal stores by ACh ( $\text{IP}_3\text{R}$  release) in 0 mM extracellular  $\text{Ca}^{2+}$  (also with CPA present). SOCC activation was indicated by a transient contraction seen with a readdition of  $\text{Ca}^{2+}$  (2 mM). To show the effects of the Icrac inhibitors, slices were pre-treated for an hour with either JS (GSK1349571A) or N117 (N11764-14-1) (maximum concentration, 10  $\mu\text{M}$ ), which were present throughout the protocol. Time matched untreated controls were included (perfusion timings table 3.10). All airways (untreated, JS (GSK1349571A) treated and N117 (N11764-14-1) treated) followed a similar pattern, representative airway tracings are shown (figure 3.30a). Summarised data is shown in figure 3.30b.

*Table 3.10 Perfusion timings designed to favour SOCC activity and to measure contraction with the addition of 2 mM  $\text{Ca}^{2+}$ . Slices were either untreated through the experiment, or pre-treated for an hour with either JS or N117 (10  $\mu\text{M}$ ), which was present throughout the protocol.*

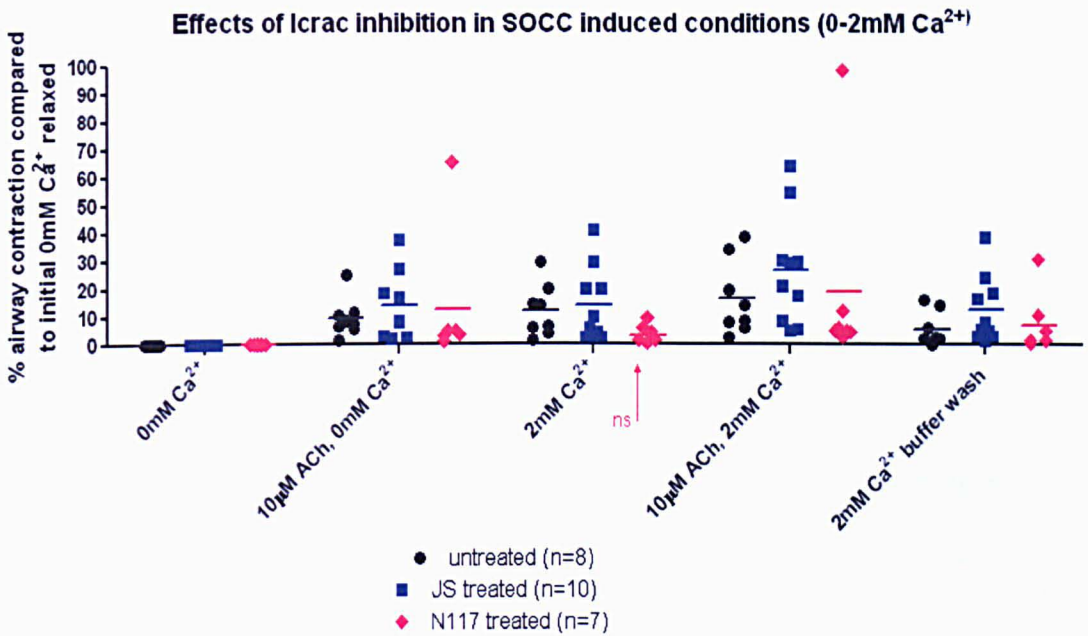
<b>Effect of Icrac inhibition in SOCC induced conditions (0 - 2 mM <math>\text{Ca}^{2+}</math>)</b>	
Time (minutes)	Perfusion
0	0 mM $\text{Ca}^{2+}$ buffer (with 1 mM EGTA, 10 $\mu\text{M}$ CPA)
3	10 ACh, 0 mM $\text{Ca}^{2+}$ (with 1 mM EGTA, 10 $\mu\text{M}$ CPA)
8	2 mM $\text{Ca}^{2+}$
11	10 $\mu\text{M}$ ACh, 2 mM $\text{Ca}^{2+}$
16	2 mM $\text{Ca}^{2+}$ buffer wash
26	end point



a) Untreated: effects of Icrac inhibition in SOCC induced conditions (0-2mM  $\text{Ca}^{2+}$ )b) JS treated: effects of Icrac inhibition in SOCC induced conditions (0-2mM  $\text{Ca}^{2+}$ )c) N117 treated: effects of Icrac inhibition in SOCC induced conditions (0-2mM  $\text{Ca}^{2+}$ )

**Figure 3.30a Effect of Icrac inhibition in SOCC induced conditions.** Representative airway tracings in 170  $\mu\text{m}$  thick lung slices. Airways were either a) untreated prior to experiments ( $n = \text{eight}$ , two pigs) or exposed to b) 10  $\mu\text{M}$  JS ( $n = \text{ten}$ , two pigs), or c) 10  $\mu\text{M}$  N117 ( $n = \text{seven}$ , two pigs) one hour prior to experiments; 10  $\mu\text{M}$  JS or 10  $\mu\text{M}$  N117 was maintained throughout the full length of the experiment protocol. For all protocols, the airway was perfused with 0 mM  $\text{Ca}^{2+}$  (with 1 mM EGTA and 10  $\mu\text{M}$  CPA added, a transient contraction was induced by 10  $\mu\text{M}$  ACh in 0 mM  $\text{Ca}^{2+}$ , a transient contraction was also seen with the readdition of 2 mM  $\text{Ca}^{2+}$ , a contraction to 10  $\mu\text{M}$  ACh in 2 mM  $\text{Ca}^{2+}$  was maintained before final wash with 2 mM  $\text{Ca}^{2+}$ . All treatment conditions produced similar airway contractile behaviour, although in general a smaller degree of contraction was observed for N117 treated slices.





**Figure 3.30b Icrac inhibition (JS and N117) did not prevent contraction in SOCC activated conditions.** Results shown for % airway contraction in 170  $\mu\text{m}$  thick lung slices. Airways were either untreated prior to experiments ( $n = \text{eight}$ , two pigs) or exposed to 10  $\mu\text{M}$  JS ( $n = \text{ten}$ , two pigs), or 10  $\mu\text{M}$  N117 ( $n = \text{seven}$ , two pigs) one hour prior to experiments; 10  $\mu\text{M}$  JS or 10  $\mu\text{M}$  N117 was maintained throughout the full length of the experiment protocol. Airway contraction is shown relative to initial airway area during 0 mM  $\text{Ca}^{2+}$  perfusion. Individual dots represent individual airways, lines indicate mean values. The Icrac inhibitor JS failed to inhibit the contraction seen to SOCC activity with the readdition of 2 mM  $\text{Ca}^{2+}$ . N117 did appear to reduce the magnitude of the contraction after readdition of  $\text{Ca}^{2+}$  however, this was not significant (as indicated with red arrow (ns), % contraction compared untreated to treated airways in 2 mM  $\text{Ca}^{2+}$ ).

Results suggested a reduction in magnitude of contraction with Icrac inhibition by N117. To provide a larger contraction in untreated airways to compare against, I next exposed the slices to extreme conditions (10 mM extracellular  $\text{Ca}^{2+}$ , 50  $\mu\text{M}$  CPA), following the same protocol as in figure 3.30. Control experiments were performed showing no contraction is induced with change from 0 mM to 10 mM extracellular  $\text{Ca}^{2+}$ . Results showed a lot of variation in degree of contraction therefore data are not shown. Although all airways showed the ability to contract, airways exposed to Icrac inhibition were less likely to maintain the contraction and

more likely to relax at the end of the protocol. Untreated airways showed continuous increasing contraction with very little final relaxation; due to the extreme conditions. Although there was a decreased mean magnitude of contraction measured in lung slices exposed to the inhibitors, although this was not significant.

Experiments in section 3.3.6 were designed to determine the contribution of SOCC to maintenance of peripheral airway contraction. Results from Icrac inhibition experiments suggest that in conditions of SR store depletion and abnormal extracellular  $[Ca^{2+}]$ , SOCC may contribute to contraction maintenance.



### 3.4 DISCUSSION

#### 3.4.1 Effects of preparation on images and contractile response of airways in mouse and pig lung slices

The techniques used for preparation of the lung slices for the work in this thesis were adapted from a method described by Bergner and Sanderson (2002). The mouse lung slices showed circular or elongated airways (figure 3.31), however, precision cut lung slice preparations from other species such as horse (Vietmeier, Niedorf et al. 2007), human (figure 3.4.1.1) and pig airways are generally web like in shape. This is most likely due to differences in anatomical structure between species, although, differences in slice preparation may contribute. For PCLS preparation in pig, horse and human, only a section of lung lobe can be filled with agarose. In preparing lung slices from mice, the entire lung is filled to  $\sim 90\%$  capacity with agarose, which may result in possible stretching of the airways prior to slicing.



**Figure 3.31** Example of a human peripheral airway in a precision cut lung slice (250  $\mu\text{m}$  thick). Tissue was obtained from patients undergoing surgical removal for cancer, only healthy tissue distanced from the tumours was used for PCLS. This image was taken from a paper by (Wohlsen, Martin et al. 2003).

In pig, slicing using the Oscillating tissue slicer was more difficult as the larger airway diameter and structures had a tendency to tear the slice. The best method for avoiding tearing was to locate a lung lobe which inflated at the very tip, which increased chance of uniform dispersion of agarose and there were no larger airways to disrupt the slice blade. The pig airways showed a greater contractile response than mouse, often to complete airway occlusion. Differences in airway sensitivity and behavioural response are most likely due to differences in receptor density and  $\text{Ca}^{2+}$  handling between the mouse and pig (discussed throughout this section).

#### **3.4.2 Contractile responses to acetylcholine, serotonin and histamine in mouse and pig**

Serotonin (5-HT) produced concentration dependent contractions in mouse peripheral airways ( $\text{EC}_{50} = 1.0 \mu\text{M}$ ). The maximum response to 5-HT ( $1 \mu\text{M}$ ) was 78% of the response to  $10 \mu\text{M}$  ACh (100%). The reduction in degree of contraction measured at 5-HT concentrations  $> 10 \mu\text{M}$  may be due to the presence of different receptor subtypes in the mouse lung producing varying effects on contraction/relaxation at different concentrations of 5-HT. Studies on various strains of rat have also confirmed that 5-HT reaches a plateau at  $1 \mu\text{M}$  (Tao, Tolloczko et al. 1999). Guinea pig PCLS gave a 5-HT  $\text{EC}_{50}$  value of 69 nM (Ressmeyer, Larsson et al. 2006). In pig no contractile response was shown for 5-HT ( $10^{-9} - 10^{-4} \text{ M}$ ); suggesting that, as in human, pig airways do not respond to 5-HT. No literature was found describing the distribution of the serotonin 5-HT<sub>2</sub> receptors in pig at the peripheral airway level. 5-HT was selected for these contractile experiments as it is known to cause bronchoconstriction in many mammalian species (Barnes, Chung et

al. 1998), (Moffatt, Cocks et al. 2004). However, there is limited evidence to suggest 5-HT directly alters human ASM tone (Cazzola and Matera 2000) but may facilitate modulation of cholinergic activation of ASM. The major source of 5-HT is platelets (Cazzola, Matera et al. 1995), although 5-HT is also localised to peripheral nerves and found in neuroendocrine cells of the respiratory tract (Barnes 2001). In human 5-HT acts on airway smooth muscle via the 5-HT<sub>2A</sub> and 5-HT<sub>1A</sub> receptor and also acts on prejunctional 5-HT<sub>3</sub> and 5-HT<sub>7</sub> receptors to facilitate increased release of ACh (Cazzola and Matera 2000; Barnes 2001). However, studies suggest species differences in the receptor subtype targeted for the direct actions of 5-HT on ASM and effects on cholinergic nerves. In mice the 5-HT induced mechanisms of bronchoconstriction may depend in part on the cholinergic parasympathetic pathway, as 5-HT induced constriction is blocked by atropine (Levitt and Mitzner 1989), (Eum, Norel et al. 1999). A recent murine model of asthma showed 5-HT release from mast cells during allergen challenge, 5-HT has also been implicated in the development of remodelling via the release of cytokines and fibrogenic mediators (Lima, Souza et al. 2007). 5-HT has also been shown to increase cilia beat frequency and particle transport speed although independent of ACh (Konig, Krain et al. 2009). Given its ability to be sourced from many non neuronal cells in the lung, 5-HT may also play a significant role in transport required to clean airways free of mucus and foreign particles.

Histamine (HA) is a known bronchoconstrictor in humans, however stimulation with HA produced no contractile effect in mouse lung slices in the concentration range of  $10^{-9}$ – $10^{-4}$  M. These findings are supported by other studies on murine PCLS (Heinz-dieter and colleagues, 1999). Little or no response was shown to HA in rat (Lulich and Paterson 1980; Van de Voorde and Joos 1998). In pig,

concentration dependent contractions were observed ( $EC_{50}$  2.2  $\mu$ M (maximum contraction of 80.2% to 100  $\mu$ M HA, compared to 10  $\mu$ M ACh response). In human PCLS an  $EC_{50}$  of 2.7  $\mu$ M was reported (Ressmeyer, Larsson et al. 2006). In horse PCLS an  $EC_{50}$  of 0.6  $\mu$ M was shown (Vietmeier, Niedorf et al. 2007). HA is synthesised and released by mast cells in the airway wall and by circulating basophils (Schroeder and MacGlashan 1997; Barnes, Chung et al. 1998). HA acting on the H1 receptor, has been shown to stimulate PI hydrolysis in ASM leading to contraction (Grandordy, Cuss et al. 1987; Hall and Hill 1988). Inhaled and intravenously administered HA contracts central and peripheral (with greater potency) airways *in vitro*; bronchoconstriction effects are increased in patients with asthma.

In mouse, concentration dependent contractions were observed for acetylcholine (ACh) ( $EC_{50}$  = 106.9 nM). These results are consistent with previous results; Sanderson and colleagues, (2002), found that concentrations of 1  $\mu$ M ACh or higher resulted in a plateau contraction in mouse lung slices. In pig, concentration dependent contractions were also shown ( $EC_{50}$  112.5 nM). In human PCLS, methacholine (MCh) (a stable analogue of ACh) gave an  $EC_{50}$  of 0.4  $\mu$ M (Wohlsen, Martin et al. 2003). Horse PCLS showed an MCh  $EC_{50}$  of 1.1  $\mu$ M (Vietmeier, Niedorf et al. 2007). ACh released from parasympathetic nerves activates postjunctional muscarinic receptors present on ASM submucosal glands and blood vessels (Mak and Barnes 1990) resulting in bronchoconstriction (Roffel, Elzinga et al. 1990), mucus secretion and vasodilation (Coulson and Fryer 2003). ACh also feeds back on prejunctional muscarinic receptors to increase or inhibit further ACh release. ACh has also been shown to increase cilia beat frequency and particle transport speed via muscarinic activation (Klein, Haberberger et al. 2009).



**Table 3.11** Comparison of  $EC_{50}$  values for acetylcholine, serotonin and histamine in human, pig and mouse airways. PCLS: Precision cut lung slices.

Agonist	Species	Tissue segment	Method for studying contractile response	$EC_{50}$	Reference
<b>Acetylcholine (ACh)</b>	mouse	distal bronchioles	PCLS	106.9 nM	Results Chapter 3
	pig	bronchi	high frequency ultrasound	15 nM	(Iizuka, Dobashi et al. 1992)
	pig	distal bronchioles	PCLS	112.5 nM	Results Chapter 3
<b>Serotonin (5-HT)</b>	mouse	bronchioles	isolated perfused and ventilated	0.7 $\mu$ M	(Held, 1999)
	mouse	bronchioles	PCLS	2.0 $\mu$ M	(Held, 1999)
	mouse	distal bronchioles	PCLS	1.0 $\mu$ M	Results Chapter 3
	pig	distal bronchioles	PCLS	no response	Results Chapter 3
	human	distal bronchioles	PCLS	no response	(Ressmeyer, Larsson et al. 2006)
<b>Histamine (HA)</b>	mouse	distal bronchioles	PCLS	no response	Results Chapter 3, (Held, 1999)
	pig	distal bronchioles	PCLS	2.2 $\mu$ M	Results Chapter 3
	human	bronchi	organ bath, isometric tension measurements	0.6 $\mu$ M	(Finney, 1985)
	human	distal bronchioles	PCLS	2.7 $\mu$ M	(Ressmeyer, Larsson et al. 2006)

### 3.4.2.i Comparison of agonist contractile responses

In comparing results from this study with previous reports it is important to acknowledge the tissue segment used and method by which response was measured. Due to easy accessibility previous studies have generally used isolated preparations of tracheal, bronchial segments and parenchymal strips in organ baths. The difference in distribution of nerves between large and small airways and the differential distribution of receptor expression at different airway levels could result in differences in response and suggests a need for pharmacological characterisation of small airway preparations in animal and human models.

Collective evidence suggests an increase in sensitivity with decreasing airway generation. One study using large and small branches of three - six generation canine bronchi found an increase in HA sensitivity correlated with a decrease in airway size (Chitano, Sigurdsson et al. 1993). In keeping with these observations, studies using rabbit airway preparations of trachea, main bronchi subsegments and parenchyma, found that distal airways were more sensitive to HA, while proximal airways showed increased sensitivity to carbachol (Armour, Black et al. 1985). MCh induced contractions using PCLS in rats also increased in sensitivity with decreased airway size (for airways less than  $35000 \mu\text{M}^2$  in size) (Martin, Uhlig et al. 1996). Another study using canine tracheal and bronchial strips in organ bath found an ACh –  $\log\text{EC}_{50}$  of 8.1, 7.1, 7.9 and 6.1 respectively in top, middle, bottom trachea and bronchi (Mapp, Chitano et al. 1989), also suggesting a decrease in sensitivity to ACh with decreasing airway order (Gray and Mitchell 1996). However, a study using trachea, main bronchi and bronchioles in rats found that small airways were less sensitive to carbachol and 5-HT than large airways (Van de Voorde and Joos 1998),

also supported by a study showing less sensitivity to 5-HT in lung parenchymal strips than in trachea and main bronchus (Cadieux, Lanoue et al. 1990). Despite the lower sensitivity, 5-HT appeared to induce a stronger contraction in small airways (Van de Voorde and Joos 1998).

There is also evidence of strain dependent differences in response. A study comparing various inbred strains of rat for intracellular signalling pathways stimulated during airway contraction found that concentration dependent increases in intracellular  $\text{Ca}^{2+}$  were seen in Fisher, Lewis and ACI strains of rat, however Fisher and ACI showed higher responses than Lewis (Eidelman, DiMaria et al. 1991; Tao, Tolloczko et al. 1999). Using video microscopy to measure rates of bronchial contraction in various strains of mouse, bronchial responsiveness was found to be greatest in A/J > Balb/C > C3H/HeJ > C57Bl/6J (Duguet, Biyah et al. 2000). These strain differences in response could be attributed to differences in muscarinic receptor density (Tao, Tolloczko et al. 1999).

In the work described in this thesis the majority of studies used C57BL/6 mice for pharmacological characterisation experiments and BALB/c mice for ovalbumin allergen sensitisation studies. Taken together these data suggest that sensitivity to contractile agents varies with species strain and regional location of the airway.

### **3.4.3 Significance of extracellular $\text{Ca}^{2+}$ and store released $\text{Ca}^{2+}$ in initiating and maintaining a contraction**

To investigate the role of extracellular  $\text{Ca}^{2+}$  in the induction and maintenance of contraction, lung slices were perfused with either 0 mM extracellular  $\text{Ca}^{2+}$  and

added EGTA (1 mM) (a divalent cation chelator with a high affinity for  $\text{Ca}^{2+}$ ) or 2 mM extracellular  $\text{Ca}^{2+}$ . Experiments showed that agonist induced contractions required  $\text{Ca}^{2+}$  release from internal stores. Inhibition of  $\text{Ca}^{2+}$  uptake into internal stores and passive outward leak of  $\text{Ca}^{2+}$  did not lead to contraction.

#### **3.4.3.i Readdition of extracellular $\text{Ca}^{2+}$ after store depletion leads to contraction**

Experiments involving SERCA pump inhibition by cyclopiazonic acid (CPA) in conditions of 0 mM and 2 mM  $\text{Ca}^{2+}$  were designed to determine whether the inhibition of  $\text{Ca}^{2+}$  uptake alone would increase cytosolic  $[\text{Ca}^{2+}]$  enough to induce contraction; no contraction was seen. In mouse, the small contraction observed with the readdition of 2 mM  $\text{Ca}^{2+}$  buffer after a prolonged state of 0 mM extracellular  $\text{Ca}^{2+}$  suggests a  $\text{Ca}^{2+}$  store depletion sensing pathway triggering extracellular  $\text{Ca}^{2+}$  entry (although this was not significant in pig).

The amount of  $\text{Ca}^{2+}$  in the SR is balanced between the depletion of  $\text{Ca}^{2+}$  via passive outward leak or agonist induced leak and repletion of  $\text{Ca}^{2+}$  via reuptake through the SERCA pump (Low, Gaspar et al. 1991). In the presence of CPA the passive outward leak of  $\text{Ca}^{2+}$  is not balanced by repletion leading to a rise in cytosolic  $\text{Ca}^{2+}$  (Henry 1994). CPA is an indole tetrameric acid metabolite of *Aspergillus* and *Penicillium* which also inhibits the ATP driven sequestration system (Shima and Blaustein 1992). CPA decreased carbachol stimulated  $\text{Ca}^{2+}$  transients in bovine tracheal single cells loaded with fura-2AM (Ethier, Cappelluti et al. 2005). Isometric tension recordings in rat tracheal SM indicated that transient contractions were induced by CPA (Henry 1994). In support of these studies, a study in isolated

rat bronchioles noted that despite the presence of atropine, a contraction was seen with the readdition of  $\text{Ca}^{2+}$ , suggesting capacitative  $\text{Ca}^{2+}$  entry through the SOCC was triggered for store refilling, this led to a rise in cytosolic  $\text{Ca}^{2+}$  levels enough to induce contraction (Sweeney, McDaniel et al. 2002). The results presented here suggest that a similar response is present in murine PCLS.

#### **3.4.3.ii Extracellular $\text{Ca}^{2+}$ is required for store refilling to maintain and repeatedly induce airway contractions.**

To investigate the role of extracellular  $\text{Ca}^{2+}$  in store refilling and  $\text{Ca}^{2+}$  release to induce airway contraction, 20 mM caffeine was chosen to open the SR located RyR. Repeated contractions were shown in 2 mM extracellular  $\text{Ca}^{2+}$ , however, only an initial contraction was shown in 0 mM  $\text{Ca}^{2+}$ . This experiment demonstrates that the contractile pathway requires  $\text{Ca}^{2+}$  derived from internal store release.

In most non excitable cell types, such as parotid acinar cells, pancreatic acini and human leukemia cells, an increase in cytosolic  $[\text{Ca}^{2+}]$  was shown to be necessary for refilling of intracellular stores (Madison, Ethier et al. 1998). However, in vascular smooth muscle,  $\text{Ca}^{2+}$  influx was shown to refill stores on store depletion, without inducing contraction (Karaki, Kubota et al. 1979; Casteels and Droogmans 1981). Furthermore, in canine smooth muscle, stores were shown to be refilled without tension development, suggesting a refilling pathway that is separate from inner cytosolic  $\text{Ca}^{2+}$  concentrations (Barnes 1985; Bourreau, Abela et al. 1991; Janssen and Sims 1993).

The physiological role of the ryanodine receptor still remains unclear. Currently adenosine diphosphate ribose (cADPR) is hypothesised to be the endogenous ligand for this receptor. RyR also shows great sensitivity to intracellular  $\text{Ca}^{2+}$  fluctuations. A rise in  $\text{Ca}^{2+}$  may activate RyR on the SR, leading to a release of  $\text{Ca}^{2+}$  resulting in contraction, referred to as  $\text{Ca}^{2+}$  induced  $\text{Ca}^{2+}$  release (CICR) (Coronado, Morrisette et al. 1994). Based on studies investigating the role of bovine and human RyR in ASM using  $\text{Ca}^{2+}$  dye fluorometry, mechanical tension and  $\text{K}^+$  currents, Tazzeo and colleagues (2008), suggest that RyR are not directly important in mediating ASM contraction or relaxation, however the RyR may direct SR  $\text{Ca}^{2+}$  towards the plasmalemma and associated  $\text{Ca}^{2+}$  extrusion mechanisms, essentially preventing SR  $\text{Ca}^{2+}$  overloading and increasing the SR  $\text{Ca}^{2+}$  buffering capacity (Tazzeo, Zhang et al. 2008). They noted that single cells loaded with fluo-4 and stimulated with caffeine showed a quick elevation in  $[\text{Ca}^{2+}]_i$  that rapidly reached a peak and then decayed, even when more caffeine was applied.

#### **3.4.4 Influence of SERCA pump inhibitors on magnitude and maintenance of contraction**

Based on previous results indicating that airway contraction was dependent on  $\text{Ca}^{2+}$  derived from internal store release, protocols were designed to determine the effect of internal  $\text{Ca}^{2+}$  store uptake inhibition on the magnitude and maintenance of contraction. The inhibition of internal  $\text{Ca}^{2+}$  store uptake did not appear to significantly influence the magnitude or ability to maintain contraction in the mouse peripheral airways. Interestingly, a significant reduction in magnitude was seen after SERCA pump inhibition using the same protocol in pig. Although the ability to



induce and sustain contraction was not affected, results in pig suggest that the supply of store released  $\text{Ca}^{2+}$  relates to the magnitude of the contraction. This information reflects species differences in internal  $\text{Ca}^{2+}$  handling.

Further studies were designed to demonstrate functional differences between the  $\text{IP}_3\text{R}$  and  $\text{RyR}$ . SERCA pump inhibition did not prevent contraction or highlight significant receptor driven differences. Previous papers measured differences in sensitivity of the  $\text{IP}_3\text{R}$  and  $\text{RyR}$  to SERCA pump inhibition, suggesting the existence of separate stores for receptor supply (Tribe, Borin et al. 1994; Golovina and Blaustein 1997; Nazer and van Breemen 1998; Janiak, Wilson et al. 2001). Interestingly, a study showed two functionally segregated SR  $\text{Ca}^{2+}$  stores within pulmonary arterial smooth muscle cells (Clark, Kinnear et al.), each served by a different SERCA pump. One in close proximity to the plasma membrane, is served by SERCA2b and releases  $\text{Ca}^{2+}$  via  $\text{RyR1}$  to promote pulmonary artery dilation. The other appears to be located centrally, is served by SERCA2a and releases  $\text{Ca}^{2+}$  via  $\text{RyR3}$ , which may in turn recruit  $\text{RyR2}$  by  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release to elicit a propagating global  $\text{Ca}^{2+}$  wave and consequent pulmonary artery constriction. Interestingly, the expression of SERCA-2 is significantly reduced in ASM from asthmatic patients, these ASM cells showed an attenuated rise in  $[\text{Ca}^{2+}]$  and delayed return to baseline (Mahn, Hirst et al. 2009). In a follow up study by the same group, healthy ASM cells treated with IL-13 and TGF- $\beta$  showed reduced SERCA-2 expression. Formoterol, the long acting  $\beta_2$ -agonist also suppressed SERCA-2 expression. Additionally, chronic  $\beta_2$ -agonist stimulation is also shown to impair cardiac relaxation via reduced SERCA-2 activity (Ryall, Schertzer et al. 2008). Collective evidence implicates a diverse network of candidates required for SR

[Ca<sup>2+</sup>] regulation and complicates further by suggesting a divided SR Ca<sup>2+</sup> store arrangement.

#### **3.4.5 Luminously continuous sarcoplasmic reticulum or separate Ca<sup>2+</sup> stores for IP<sub>3</sub>R and RyR release?**

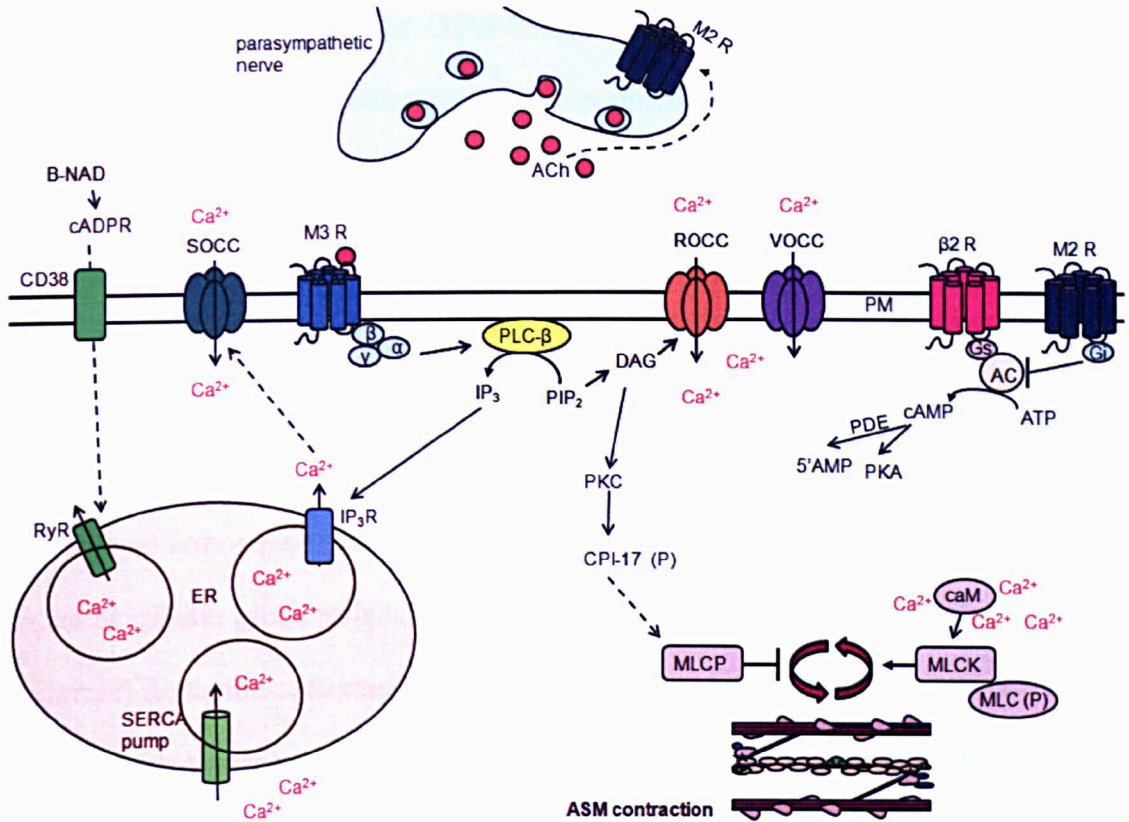
In order to investigate the functional roles of the SR located IP<sub>3</sub>R and RyR and to determine whether separate Ca<sup>2+</sup> stores supply these receptors, I hypothesised i) that the IP<sub>3</sub>R shares a common Ca<sup>2+</sup> store with the RyR for release and has a main role in contraction maintenance and ii) that the RyR plays a role in [Ca<sup>2+</sup>] regulation between the SR and cytoplasm and may therefore have access to separate SR Ca<sup>2+</sup> stores. Numerous studies investigating the structural arrangement of the SR have suggested the all or none physiological response observed to pharmacological agents may be explained by multiple separate Ca<sup>2+</sup> store elements (Parker and Ivorra 1990; Bootman, Berridge et al. 1992; Hirose and Iino 1994). Exhaustion of response was measured via each receptor using conditions to deplete the store. Stimulation of the IP<sub>3</sub>R for release by ACh was unable to stimulate contraction following depletion of stores via RyR release, suggesting a common pool. Despite prolonged store depletion and following IP<sub>3</sub>R release, contractions were seen with RyR release, which could be interpreted as a structurally separate Ca<sup>2+</sup> store for the RyR release pathway. However, against this hypothesis was the observation of a second contraction following repeated exposure to ACh. Other studies suggested after the SR has been depleted by a maximal response to IP<sub>3</sub>R stimulation, a substantial response to caffeine persists, which has previously been interpreted as the arrangement of separate stores for both receptors (Yamazawa, Iino et al. 1992;

Janiak, Wilson et al. 2001). Evidence to suggest a heterogeneous SR structure comes from studies in smooth muscle showing that the peripherally located SR may be depleted more readily than the SR located near the center of the cells (Devine, Somlyo et al. 1972). Calsequestrin was shown to be clustered at sites within the peripheral SR, this location was also dense in IP<sub>3</sub>R (Villa, Podini et al. 1993).

In contrast there is evidence to suggest the SR is a single lumenally continuous structure containing both IP<sub>3</sub>R and RyR in which Ca<sup>2+</sup> can freely diffuse throughout. A study using pancreatic acinar cells noted free diffusion of Ca<sup>2+</sup> throughout the store by the refilling of Ca<sup>2+</sup> in the apical region despite the Ca<sup>2+</sup> being introduced at the opposite end of the cell (Mogami, Nakano et al. 1997). Using confocal microscopy to monitor the movement of Ca<sup>2+</sup> and small fluorescent probes in the ER lumen of pancreatic acinar cells, diffusion in to small apical ER extensions was observed suggesting Ca<sup>2+</sup> release from the apical ER is replenished from the bulk of the rough ER at the base (Park, Petersen et al. 2000). Studies in ventricular myocytes using fluorescent recovery after photo bleaching (FRAP) observed dye re-equilibration in the SR, also suggesting a continuous structure (Wu and Bers 2006). Studies in cultured cell types using photo bleaching of a Ca<sup>2+</sup> store located green fluorescent protein (GFP) also noted that the GFP was able to move freely throughout (Montero, Alvarez et al. 1997; Subramanian and Meyer 1997; Dayel, Hom et al. 1999). These studies suggest sensitivity differences may arise by regulation of IP<sub>3</sub>R and RyR opening by agonist concentration acting on the cytoplasmic portion of the receptor. As SR [Ca<sup>2+</sup>] declines, the receptors sensitivity to their cytoplasmic ligand is reduced perhaps more so for one receptor than the other creating the appearance of functional differences; this could then be interpreted as structurally different stores. Furthermore, regions with higher densities of channels, pumps or luminal Ca<sup>2+</sup>

binding proteins in the SR may result in sites with differing sensitivities to agonists/inhibitors (McCarron and Olson 2008); giving the impression of store subcompartments existing.

Conflicting theories in the literature have lead to contradicting interpretations. The data presented here do not provide a conclusive answer regarding the identity of these compartments in ASM. Two possibilities still exist, namely the existence of two spatially separate but partly coupled stores (figure 3.32), or incomplete emptying of a single store following either RyR or ACh stimulation. Differences between mouse and pig in response to the same protocols may reflect species differences in receptor densities at sites on the SR; influencing signaling for channels/ pumps for refilling and  $\text{Ca}^{2+}$  release. A time matched control showing the behaviour of the airways with prolonged 0mM extracellular  $\text{Ca}^{2+}$  prior to agonist stimulation should have been included to interpret whether the exhaustion of the response is due to depletion of intracellular stores via separate stores or simply global depletion of stores due to prolonged 0mM  $\text{Ca}^{2+}$  exposure. Such controls will be included in future studies.



**Figure 3.32** The relationship for  $\text{Ca}^{2+}$  stores within the SR supplying the RyR and  $\text{IP}_3\text{R}$  pathways remains unclear. Further investigation is required to determine whether i) the SR is luminously continuous or ii) made up of separate  $\text{Ca}^{2+}$  stores which separately supply the RyR and  $\text{IP}_3\text{R}$  pathways or iii) consists of stores that partially share a common supply of  $\text{Ca}^{2+}$  for release via the RyR and  $\text{IP}_3\text{R}$ . ER: endoplasmic reticulum; PM: plasma membrane; M2 R: muscarinic cholinergic receptor type 2; M3R: muscarinic cholinergic receptor type 3; MLCK: myosin light chain kinase; MLCP: myosin light chain phosphatase; myosin-P: phosphorylated myosin light chains; RyR: ryanodine receptor, SERCA: sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase,  $\text{IP}_3$ : inositol triphosphate,  $\text{IP}_3\text{R}$ :  $\text{IP}_3$  receptor, PLC- $\beta$ : phospholipase C, DAG: diacylglycerol,  $\text{PIP}_2$ : phosphatidylinositol 4,5-bisphosphate.  $\beta_2$  R:  $\beta_2$ -adrenergic receptor; AC: Adenyl cyclase; cAMP: cyclic adenosine monophosphate; PDE: phosphodiesterase; PKA: protein kinase A. Adapted from (Dietrich, Chubanov et al. 2006; Pelaia, Renda et al. 2008).

### 3.4.6 Role of Voltage Operated Calcium Channels (VOCC) in peripheral airway contraction

The data shown here demonstrate that the L-type VOCC antagonist nifedipine did not significantly reduce contractile responses to ACh in peripheral

mouse or pig airways. The L-type VOCC has remained the best characterised pathway of  $\text{Ca}^{2+}$  entry, however, varied results have been produced in mouse, rat, guinea pig and human for experiments attempting to inhibit agonist induced contraction. Using a mouse bronchial ring preparation, when pretreated with nifedipine, the maximal contractile response to MCh was lowered by 51.5% ( $\text{EC}_{50}$  0.9  $\mu\text{M}$ ), suggesting VOCC may play a role at the bronchial level in mouse (Liu, Yang et al. 2006). The results of the data shown here suggest that nifedipine does not induce relaxation at the peripheral airway level; this is supported by other lung slice models (Perez and Sanderson 2005). Clinical studies using nifedipine and other L-VOCCs blockers found overall insignificant effects on contraction (Ferrari, Olivieri et al. 1989); suggesting that voltage gated  $\text{Ca}^{2+}$  entry pathways are not important in asthma bronchoconstriction (Barnes 1985). A likely explanation for the lack of inhibition by L-VOCCs inhibitors in the peripheral airway level is the presence of alternative  $\text{Ca}^{2+}$  entry pathways such as T-type-VOCC, store operated (SOCC) (discussed in 3.4.7) and receptor operated calcium channels (ROCC) on the plasma membrane.

#### **3.4.7 Airway contraction inhibited by selected $\text{Ca}^{2+}$ binding agents and Store Operated Calcium Channel (SOCC) inhibitors**

Given the previous results showing a lack of VOCC contribution to airway contraction/relaxation; the next set of experiments were designed to determine whether agonist induced maintenance of contraction is dependent on store refilling via the SOCC (1.2.3).



The results presented here showed that 2-APB provided some relaxation at concentrations above 10  $\mu\text{M}$ , although more so for mouse (1 mM, 81% relaxation) than pig (1 mM, 34% relaxation). 2-APB within a range of 1 - 100  $\mu\text{M}$  has been shown to exhibit  $\text{Ca}^{2+}$  signal inhibition in numerous cell types acting via the  $\text{IP}_3\text{R}$  (Bootman, Collins et al. 2002). However, studies using permeabilised cells, cell membranes and intact cells suggest that 2-APB may be an inconsistent  $\text{IP}_3\text{R}$  inhibitor and that the dose used is important (Maruyama, Kanaji et al. 1997). Its variable effects are also suggested to be due to the differential action of different  $\text{IP}_3\text{R}$  isoforms. Hence whilst isoform type 2  $\text{IP}_3\text{R}$  is insensitive to 2-APB effects, for type 1 and 3  $\text{IP}_3\text{R}$  obvious effects are shown. For these experiments the choice of  $\text{IP}_3$  antagonist was limited. Other tools have been used to study  $\text{Ca}^{2+}$  signalling but again have non specific actions. Heparin is commonly used however, its multiple actions include uncoupling G-protein signalling and activating RyR (Ehrlich, Kaftan et al. 1994; Jonas, Sugimori et al. 1997; Taylor and Broad 1998). Xestospongins have been described as a slow acting membrane permeant  $\text{IP}_3\text{R}$  antagonist to inhibit  $\text{Ca}^{2+}$  signalling (Gafni, Munsch et al. 1997); however its mechanism is not clear. In addition to decreasing the release of internal  $\text{Ca}^{2+}$  stores, 2-APB may also inhibit the SOCC that replenish  $\text{Ca}^{2+}$  stores (Bootman, Collins et al. 2002), in the high  $\mu\text{M}$  range ( $> 100 \mu\text{M}$  2-APB) (Dobrydyneva and Blackmore 2001; Flemming, Xu et al. 2003).

In mouse nickel ( $\text{Ni}^{2+}$ ) (an inhibitor of  $\text{Ca}^{2+}$  influx through SOCC) provided relaxation at concentrations  $> 100 \mu\text{M}$  (1 mM, 36% relaxation). In pig relaxation was only shown at maximum concentration (1 mM, 12 % relaxation). A study in bovine trachea cells loaded with Fura-2 AM noted that  $\text{Ni}^{2+}$  (2 mM) maximally decreased resting  $[\text{Ca}^{2+}]_i$  but had no significant effect on refilling; it was further suggested that

the channels through which  $\text{Ca}^{2+}$  passed to the inner cytosol to maintain resting  $[\text{Ca}^{2+}]_i$  levels had a different sensitivity to  $\text{Ni}^{2+}$  than the channels reserved for refilling or agonist sensitive stores (Madison, Ethier et al. 1998).

Lanthanum provided relaxation in mouse (1 mM, 68% relaxation). However, in pig very little relaxation was seen (1 mM, 5% relaxation). The lanthanides, lanthanum ( $\text{La}^{3+}$ ) and gadolinium ( $\text{Gd}^{3+}$ ) inhibit  $\text{Ca}^{2+}$  permeable cation channels and have been used for the characterisation of Transient Receptor Potential (TRP) channels (Halaszovich, Zitt et al. 2000). Lanthanum's charge provides a high affinity for surface membrane anionic sites such as those normally occupied by  $\text{Ca}^{2+}$  and its' large hydrated ionic radius prevents cell entry, creating a specific  $\text{Ca}^{2+}$  channel inhibitor, although at higher concentrations it has been shown to inhibit plasma membrane  $\text{Ca}^{2+}$  pumping (Kwan, Takemura et al. 1990; Putney, 2001).

One explanation for SOCC diversity in different cell types may be attributed to the differential expression of various TRPC channel subunits. TRPC1 is known to be sensitive to blocking by 0.02 – 1 mM  $\text{Gd}^{3+}$  or 0.01 - 1 mM  $\text{La}^{3+}$  (Zitt, Zobel et al. 1996; Sinkins, Estacion et al. 1998; Liu, Wang et al. 2000). TRPC4 and TRPC5 have been shown to be stimulated by  $\text{La}^{3+}$  and  $\text{Gd}^{3+}$  (Schaefer, Plant et al. 2000; Strubing, Krapivinsky et al. 2001; Allakhverdi, Smith et al. 2007). A possible TRPC, 25 pS non selective cation channel was previously identified with sensitivity to 1 mM  $\text{La}^{3+}$ ,  $\text{Gd}^{3+}$  and 100  $\mu\text{M}$  SKF-96365 and activated by leukotrienes in human bronchial smooth muscle (Snetkov, Hapgood et al. 2001). A 25 pS non selective cation channel in bovine TSM was also identified that contributed to  $\text{Ca}^{2+}$  store refilling and cytosolic  $\text{Ca}^{2+}$  concentrations (Helli and Janssen 2008), with CPA induced  $\text{Ca}^{2+}$

entry that was resistant to nifedipine. Previous work by our group suggests that human ASM expresses TRPC1,3,4 & 6.

I next explored the effects of Icrac inhibition on ASM tone. Initial inhibitory dose responses for JS (GSK1349571A) and N117 (N11764-14-1) in the presence of ACh suggested that in physiological conditions SOCC do not contribute to airway contraction maintenance. Therefore I measured airway contractile behaviour in conditions designed to favour SOCC activity; using one hour pre-treatment with the Icrac inhibitors, which were also maintained throughout the experiment with a change from 0 mM  $\text{Ca}^{2+}$  (10  $\mu\text{M}$  CPA present) to 2 mM extracellular  $\text{Ca}^{2+}$ . Treatment with N117 did appear to reduce the contractile response (albeit not significantly). Given the ineffectiveness of the majority of the SOCC inhibitors used in this study, it appears that the contribution of SOCC entry for ASM contraction in physiological conditions is low although in conditions of store depletion or abnormal  $[\text{Ca}^{2+}]$ , SOCC appear to contribute to SR refilling and release for maintenance of contraction. Taken together these data argue for a role for SOCC in regulating the intracellular  $\text{Ca}^{2+}$  store, but argue against short term regulatory effects on ACh contractile response, under normal physiological conditions.

There is limited data to suggest a major role for SOCC in contributing to skeletal and smooth muscle tone based on abnormal regulation resulting in functional consequences; although SOCC co-existing with non-store operated  $\text{Ca}^{2+}$  influx pathways may compensate for a lack of functional STIM1 and Orai1. Defects in  $\text{Ca}^{2+}$  handling in vascular smooth muscle cells are among the candidate mechanisms leading to hypertension. Studies on aortic rings from stroke-prone spontaneously hypertensive rats noted increased expression of STIM1 and Orai1,

which contributed to abnormal vascular function (Giachini, 2009) such as increased force development after depletion of  $\text{Ca}^{2+}$  stores (Kanagy, Ansari et al. 1994). In human, further normoxic contraction of the ductus arteriosus at birth is related to  $\text{Ca}^{2+}$  entry through SOCC and the ability to increase  $\text{Ca}^{2+}$  sensitisation (based on experiments inhibiting Rho kinase activity with Y-27632, which removed the normoxic contraction). Such findings have therapeutic implications for the treatment of congenital heart disease (Hong, Hong et al. 2006). Regarding the inclusion of TRPC channels as SOCC; there is evidence for SOCC involvement in Duchenne Dystrophy, displaying TRPC related abnormal  $\text{Ca}^{2+}$  homeostasis in skeletal muscle (Vandebrouck, Martin et al. 2002). TRPC6 channels play a role in contractility of aortic and vascular smooth muscle (Targos, Baranska et al. 2005). They are expressed in cells responding to hydrostatic pressure changes and suggested to mediate pressure induced responses. TRPC6 channels are implicated in smooth muscle proliferation in patients with idiopathic pulmonary arterial hypertension (Yu, Fantozzi et al. 2004) and suggested to generate myogenic tone in arteries (Welsh, Morielli et al. 2002; Beech, Muraki et al. 2004). TRPC6 knockout mice show increased arterial blood pressure, increased arterial tone and enhanced agonist and DAG induced current in smooth muscle (the unexpected increased  $\text{Ca}^{2+}$  mediated response resulted from a compensated increase in the expression of TRPC3) (Freichel, Vennekens et al. 2004; Dietrich, Mederos y Schnitzler et al. 2005; Dietrich, Mederos et al. 2005). Mouse models globally targeting STIM1 and Orai1 result in early death postnatally (Feske, Gwack et al. 2006; Feske 2009; Picard, McCarl et al. 2009). Although, studies investigating the outcome of individual deletions in mouse models typically focus on T cells, mast cells and implications for the immune system rather than resulting skeletal or smooth muscle development.

Mutations in STIM and Orai result in rare human hereditary immunodeficiency syndromes. Patients display a loss of T cell cytokine expression, store operated  $\text{Ca}^{2+}$  entry and CRAC current (Partiseti, Le Deist et al. 1994; Le Deist, Hivroz et al. 1995; Le Deist, Hivroz et al. 1995; Feske, Draeger et al. 2000; Feske, Draeger et al. 2000; Feske, Prakriya et al. 2005; Feske, Gwack et al. 2006; Feske 2009; McCarl, Picard et al. 2009; Picard, McCarl et al. 2009). Evidence suggests that STIM1 and Orai1 are the predominant family members responsible for store operated  $\text{Ca}^{2+}$  entry in human T cells. The predominant clinical phenotype consists of severe T-cell immunodeficiency, recurrent viral, fungal and bacterial infections, enamel calcification defects and in specific STIM1 deficiency, autoimmunity is seen (Feske 2009). Congenital myopathy is also associated; consistent with the roles of STIM1 and Orai1 in SOCC in skeletal muscle (Rosenberg 2009; Dirksen, 2009) and STIM1 in myoblast differentiation (Feske 2009). This myopathy eventually leads to severe chronic pulmonary disease with increased mucus retention in the airways (McCarl, Picard et al. 2009).

The data presented in this chapter demonstrates that precision cut mouse and pig lung slices provide a robust model to study mechanisms underlying contraction and relaxation of airway smooth muscle. Results highlight interesting species differences in agonist and inhibitor sensitivity, with the porcine system sharing similar pharmacology to human airways. The next chapter describes the application of this technique to a sensitised mouse model of airway inflammation.

## **CHAPTER FOUR RESULTS**

### **IL-33 AS A MEDIATOR OF AIRWAY HYPER-RESPONSIVENESS IN A MURINE MODEL OF AIRWAY INFLAMMATION**



## 4. CHAPTER FOUR

### 4.1 INTRODUCTION

Asthma is associated with airway hyper-responsiveness, airway remodelling, eosinophilic airway inflammation and mucus hyper-secretion. The response has been linked in many model systems to up regulation of the  $T_H2$  cytokines, IL-4, IL-5, IL-9 and IL-13 (Robinson, Hamid et al. 1992) (discussed in 1.1). The best approach to gain understanding of the pathways involved in asthma and potential drug therapies would be to perform experiments on human patients with asthma; such studies provide practical difficulties. Animal models provide an alternative to gain understanding of the progression of the mechanisms involved in allergic airway disease. However, because asthma is a complex multifactorial disease, it is not possible to develop a single animal model that can replicate chronic human asthma (Nials and Uddin 2008); therefore the animal models developed aim to mimic specific features of the disease. Many models of acute and chronic airway inflammation have now been established. Due to accumulated knowledge of mouse immunology, access to the full genomic sequence and gene targeted mice, mouse models have proved particularly useful and seemed an obvious direction for experiments with our PCLS technique already set up for murine lungs.

The process for induction of airway inflammation in sensitised models involves systemic priming of the immune system against an antigen, followed by challenge, a triggering of the allergic reaction by a second application of the antigen in the airways. Variations in dose of antigen and form of administration used can influence the manifestation. The most natural allergen models involve challenge with

house dust mite, pollen or cockroach extracts by intranasal or inhaled administration. Previously we had explored differences in airway contractile behaviour using an acute mouse model of house dust mite extract exposure via intranasal administration (data not shown) and found no major differences in airway sensitivity to 5-HT or MCh using the lung slice technique; difficulties in the allergen reaching the distal airways were debated. For our studies, ovalbumin (OVA) derived from chicken egg white protein was our choice of allergen, given its well established use and ability to induce robust, allergic inflammation in many animals (Nials and Uddin 2008). Initial sensitisation involved intraperitoneal injection with OVA (in combination with an adjuvant, aluminium hydroxide), further challenges required aerosolised OVA (described in 2.1). In these experiments the BALB/c strain of mouse was chosen for antigen challenge, as this strain develops a strong  $T_H2$  biased response (Boyce and Austen 2005). C57/BL and A/J strains have also been used successfully (Kumar, Herbert et al. 2008).

Using OVA challenge in BALB/c mice, we aimed to create a model of airway disease with such specific features as AHR and chronic exposure induced airway remodelling; to replicate these aspects of human asthma. In this chapter, the distal airway structural changes observed and contractile behaviour measured using the PCLS technique provided a fascinating insight into the ramifications of such allergen exposure for this mouse model.

The pathway involving the  $T_H2$  inflammatory response is the particular focus in this chapter. After initial allergen encounter naïve T cells are reported to undergo differentiation leading to the production of  $T_H2$  cytokines, further challenges result in inflammatory cell recruitment to the airways and progression of the disease. Table

4.1 provides a list some of the cytokines involved in this inflammatory process that were considered for investigation in this chapter.

**Table 4.1 Cytokines involved in the airway inflammatory process.** All cytokines listed were initially used individually for treatment of naïve WT mouse lung slices, to identify any specific influences of these cytokines on airway contraction behaviour.

T <sub>H</sub> 2 Cytokines		Summary
<b>IL-4</b>	Drives differentiation of naïve T <sub>H</sub> 0 cells to T <sub>H</sub> 2 cells, which secrete IL-4, IL-5, IL-9, IL-13 but not interferon (O'Garra and Arai 2000; Murphy and Reiner 2002). Antigen induced allergic inflammation was shown to be decreased in IL-4 deficient mice (Brusselle, Kips et al. 1994). Anti-IL-4 Ab prior to antigen immunisation prevented the development of antigen induced airway inflammation, however the same administration after immunisation and prior to antigen inhalation was not effective in preventing the inflammatory response (Coyle, Le Gros et al. 1995).	Required for initial differentiation of antigen specific T <sub>H</sub> 2 cells.
<b>IL-5</b>	Originally identified as a T cell derived cytokine that triggers activated B cells for terminal differentiation into immunoglobulin producing cells (Takatsu, Takaki et al. 1994). Major maturation and differentiation mediator for eosinophils, which are involved in fibrosis by producing a fibrogenic factor (TGF- $\beta$ 1) (Koike and Takatsu 1994; Rothenberg and Hogan 2006). Studies have shown increased expression of IL-5 mRNA in bronchial biopsies of asthmatic patients (from CD4+ T cells) (Robinson, Hamid et al. 1992). Forced expression of IL-5 in transgenic mice leads to eosinophil infiltration (Lee, McGarry et al. 1997). Anti-IL-5 mAb was shown to inhibit antigen induced AHR and eosinophil infiltration in mice and guinea pig airways (Akutsu, Kojima et al. 1995). Antigen sensitised IL-5 deficient mice show lack of bronchial hyper-reactivity and eosinophilia (Foster, Hogan et al. 1996). Anti-IL-5 Ab treatment did not reduce asthmatic symptoms and airway reactivity in patients with mild asthma (Leckie, ten Brinke et al. 2000). Although, anti-IL-5 treatment in mild atopic asthmatics has been shown to reduce ECM deposition in the bronchial subepithelial basement membrane. Fibrosis is abolished in sensitised and allergen exposed IL-5 receptor null mice, but increased in IL-5 transgenic mice. Treatment with anti-IL-5 Ab in WT mice almost completely abolished subepithelial and peribronchial fibrosis in sensitised mice (Tanaka, Komai et al. 2004).	Possible role in the late asthmatic response. Key mediator for eosinophils, involved in fibrosis.
<b>IL-9</b>	Expression of IL-9 mRNA was increased in bronchial biopsy samples of asthmatics (Shimbara, Christodoulopoulos et al. 2000). Possible genetic linkage to	Role in inflammatory process via

	AHR (Nicolaidis, Holroyd et al. 1997). Suggested that IL-9 may play a role in goblet cell hyperplasia and mast cell development based on experiments using IL-9 deficient mice (Townsend, Fallon et al. 2000). Primary ASM cells express a functional IL-9R <i>in vitro</i> , further more ASM bundles from atopic asthmatics showed increased expression of IL-9R (Gounni, Hamid et al. 2004). Stimulation of IL-9R on ASM showed increased expression of CCL11 via STAT3 signalling, showing a role for enhanced eosinophil chemotaxis (Yamasaki, Saleh et al.).	chemotaxis.
<b>IL-13</b>	Antigen challenge in IL-13 deficient mice failed to show AHR and mucus production despite the presence of IL-4, IL-5 and airway inflammation (Walter, McIntire et al. 2001). IL-13 is consistently over expressed in the lungs of asthmatic patients (Wills-Karp 2004) and is reported to contribute to the T <sub>H</sub> 2 mediated inflammatory response via its ability to stimulate gene expression of adhesion molecules, chemokines and metalloproteinases. Inhibition of IL-13 <i>in vivo</i> by administration of the soluble IL-13 $\alpha$ 2-Ig or through IL-13 gene targeting in mice either prevents or reverses established mucus cell changes (Grunig, Warnock et al. 1998; Wills-Karp, Luyimbazi et al. 1998; Walter, McIntire et al. 2001). IL-13 signalling pathway members IL-4R $\alpha$ and STAT6 have been shown to be involved in mucus cell changes (Kuperman, Schofield et al. 1998). IL-4R $\alpha$ chain polymorphisms may play a role in asthma susceptibility (IL-33 was also linked) (Hoffjan and Ober 2002).	Acts on epithelial and smooth muscle cells. Role may be independent of other T <sub>H</sub> 2 cytokines. Role in AHR.

Pro-inflammatory Cytokines		Summary
<b>IL-17A</b>	Expressed in a subpopulation of CD4 <sup>+</sup> T cells, namely T <sub>H</sub> 17 cells, involved in autoimmune disease (Weaver, Harrington et al. 2006). Induce expression of cytokines and chemokines including IL-6, granulocyte macrophage colony stimulating factor and induce IL-8 from fibroblasts (Linden 2001; Moseley, Haudenschild et al. 2003; Kawaguchi, Adachi et al. 2004). Mediate migration of neutrophils, but not eosinophils, maybe involved in severe asthma, as neutrophils are a hallmark (Jatakanon, Uasuf et al. 1999).	Possible link between activation of T cells and recruitment of neutrophils.
<b>IL-25</b>	Produced by activated T <sub>H</sub> 2 cells (Fort, Cheung et al. 2001) and mast cells (Ikeda, Nakajima et al. 2003). Systemic administration of protein (Fort, Cheung et al. 2001), or expression by transgene (Pan, French et al. 2001) induces IL-4, IL-5 and IL-13 production from undefined non-T, non-B cells, resulting in blood eosinophilia, increased serum IgE levels and pathological changes in lung and other tissues (Tamachi, Maezawa et al. 2006). IL-25 mRNA was expressed in lung after antigen inhalation, neutralisation of IL-25 by IL-25R decreased antigen-induced eosinophil and	May play a role in amplifying/ prolonging T <sub>H</sub> 2 mediated inflammation in asthma.

	CD4+ T cell recruitment into the airways. Enhanced expression in asthmatic patient airways (Letuve, Lajoie-Kadoch et al. 2006).	
<b>Tumour necrosis factor-<math>\alpha</math> (TNF-<math>\alpha</math>)</b>	Leukocyte recruitment through up regulation of adhesion molecules on endothelial cells (Babu, Davies et al. 2004). Induction of cytokines, chemokines involved in asthma (Babu, Davies et al. 2004). Increased levels found in BAL and biopsy samples in asthmatic patients (Thomas, Yates et al. 1995; Keatings, Collins et al. 1996). Inhalation of TNF- $\alpha$ causes AHR and increased sputum neutrophils (Thomas, Yates et al. 1995). Anti-TNF- $\alpha$ Ab reduce endotoxin and IL-18-induced changes in airways in rodent models (Kips, Tavernier et al. 1992; Kumano, Nakao et al. 1999). Treatment with the TNF- $\alpha$ antagonist 'etanercept' is shown to reduce AHR and asthma symptoms (Berry, Hargadon et al. 2006).	Role in pathogenesis of severe asthma.
<b>IL-33</b>	IL-1 like cytokine (Schmitz, Owyang et al. 2005). IL-33's receptor, ST2 is an IL-1R- related protein expressed on T cells and mast cells (Trajkovic, Sweet et al. 2004). ST2 may have a possible role in T <sub>H</sub> 2 responses in asthma (Lohning, Stroehmann et al. 1998). Produced by smooth muscle cells, epithelial and dendritic cells, IL-33 drives production of IL-5 and IL-13 from T-cells (Schmitz, Owyang et al. 2005). Stimulates primary human mast cells to produce proinflammatory cytokines and chemokines (Allakhverdi, Smith et al. 2007). Acts with TSLP to accelerate maturation of CD34 <sup>+</sup> mast cell precursors and induce secretion of T <sub>H</sub> 2 cytokines and T <sub>H</sub> 2-attracting chemokines (Allakhverdi, Smith et al. 2007). Treatment of mice with IL-33 induces expression of high levels of IL-4, IL-5 and IL-13 in various tissues, with eosinophilia and increased IgE and IgA. IL-33 treated mice show severe inflammatory lesions of the lungs and digestive tract with eosinophilic infiltrates, epithelial cell hyperplasia and increased production of mucus (Schmitz, Owyang et al. 2005). Human IL-33 mRNA is expressed in airway epithelial cells, bronchial smooth muscles and smooth muscle of the coronary and pulmonary arteries (Schmitz, Owyang et al. 2005).	The IL-33/ST2 interaction may play a role in induction of airway inflammation involving mast cells.

#### 4.1.1 AIMS

This chapter aimed to explore the pathogenesis of hyper-reactivity implicated in airway inflammatory disease by studying mechanisms involved in inflammatory

mediated increased airway smooth muscle contraction in a murine model of airway inflammation.

## **4.2 METHODS**

Detailed methods for the preparation of lung slices, use of an oscillating tissue slicer, measuring contractile response and staining of slices are provided in the materials and methods in 2.1.3. Use of the cryostat slicer for mouse lung tissue slicing and H&E staining is described in 2.1.4. Protocols for cytokine incubation and ovalbumin sensitisation are also outlined in 2.1.

### **4.2.1 Perfusion timing protocols specific to Chapter Four Results**

For image capture of the airway contraction, the lung slices were held in place on a microscope slide attached to a chemical perfusion system. For most experiments shown, differences in magnitude of contraction were measured to a maximum concentration of 10  $\mu$ M methacholine (MCh) for five minutes followed by a ten minute wash with buffer (table 4.2). A dose response to MCh was included to view changes in airway sensitivity (table 4.3). All chemicals were perfused at a constant flow rate (2 ml/minute) over the lung slice during real time video image capturing of the airway contraction.



*Table 4.2 Perfusion timings for contraction to 10 $\mu$ M methacholine (MCh) with wash timings for airway relaxation measurement.*

<b>10 <math>\mu</math>M methacholine (MCh) for measurement of airway contraction</b>	
Time (minutes)	Perfusion
0	2 mM Ca <sup>2+</sup> buffer
1	10 $\mu$ M MCh
6	buffer wash out
11	measurement of relaxation (five minutes)
16	end point: measurement of relaxation (ten minutes)

*Table 4.3 Methacholine (MCh) dose response perfusion timings for buffer and increasing agonist concentrations until relaxation of the airway with wash buffer.*

<b>Contractile agonist dose response</b>	
Time (minutes)	[MCh]
0	2 mM Ca <sup>2+</sup> buffer
1	0.001 $\mu$ M
6	0.01 $\mu$ M
11	0.1 $\mu$ M
16	0.5 $\mu$ M
21	1 $\mu$ M
26	10 $\mu$ M
31	100 $\mu$ M
36	buffer wash out
46	end point

#### 4.2.2 Statistical Analysis

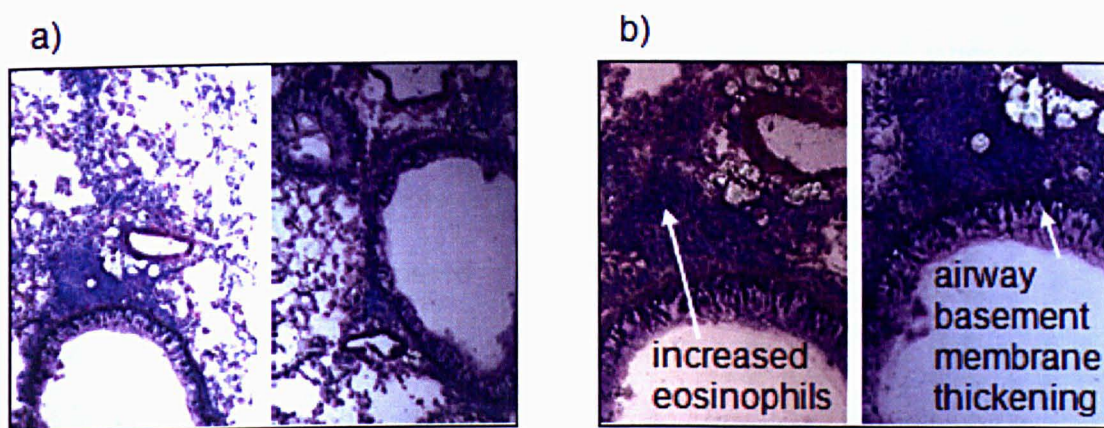
For all results shown in this chapter, graphed values are derived from the initial airway lumen area measurements for individual mice (n= number). Values were then expressed as percentage contraction from the initial baseline values (at buffer perfusion) when the airway is relaxed. For all figures shown in this chapter,

statistical significance was assessed using the Mann Whitney test; used to acknowledge median values (rather than mean) and rank distribution of the values between control and treatment groups. For all scatter dot plot graphs each dot represents an individual airway contraction measurement from an individual mouse, red lines indicate median values in each group. Dose response experiments were analysed with the PRISM software Graphpad 5.02 using analysis tools for dose response for the log of the agonist against the response for variable data. In this chapter  $EC_{50}$  measurements with confidence intervals (CI) were calculated to provide a measure of differences in airway sensitivity between control and sensitised mice or treated slices, when exposed to the same drug concentration protocols (MCh dose response).

### 4.3 RESULTS

#### 4.3.1 Structural remodelling, inflammatory cell infiltration and responsiveness of the airways is increased with repeated ovalbumin allergen exposure

Cardiac lobes from either PBS or chronic OVA sensitised airways were frozen in liquid nitrogen and cut to 20  $\mu$ m thick slices using a cryostat tissue slicer. Staining revealed increased eosinophils in the OVA sensitised airways compared to PBS treated groups (figure 4.1).

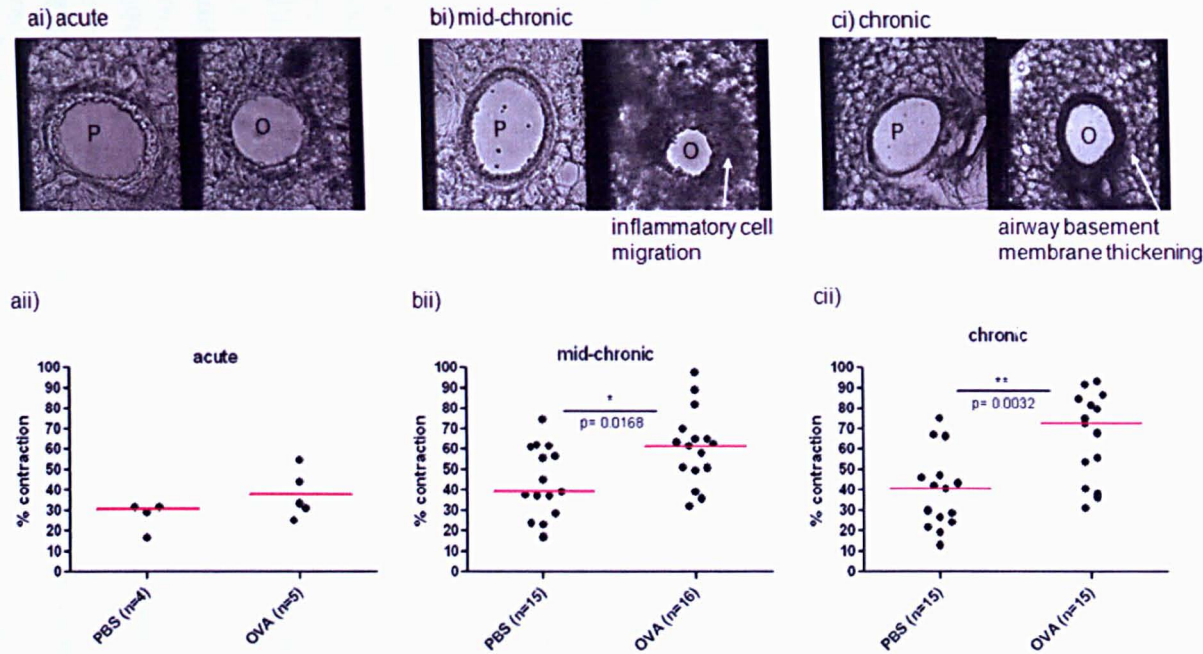


**Figure 4.1 Airway remodelling with increased eosinophils in OVA sensitised lung tissue.** Representative H & E staining of 20  $\mu$ m thick a) PBS control ( $n$  = ten airways from ten mice) and b) OVA chronic sensitised mouse lung slices ( $n$  = ten airways from ten mice), taken at 200x magnification using a Nikon DIAPHOT 300 microscope. Increased eosinophils and structural changes to the airway wall (arrows), particularly basement membrane thickening are visible by eye.

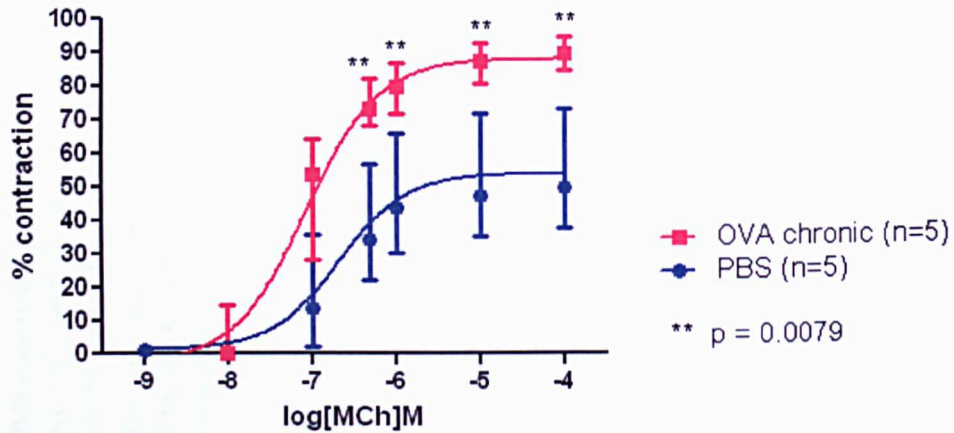
Previous results from the MRC laboratory, Cambridge, had revealed significantly increased airways resistance to MCh in OVA chronic sensitised airways compared to PBS treated airways (data not shown, experiment by Jillian Barlow). I then designed experiments to directly measure the influence of OVA sensitisation on airway sensitivity at the distal airway level using the PCLS technique. In contraction experiments shown 'n' equates to number of airways from individual mice, i.e.  $n$  =

15 equates to 15 airways from 15 mice. I initially designed dose response experiments to MCh and 5-HT for chronic OVA sensitised and matched non-sensitised mice in view of highlighting increased airway sensitivity in the allergen exposed mice. Concentration curve experiments to MCh were run 24 hours after mouse sacrifice and lung excavation; during experiments an increase in airway sensitivity was obvious by eye (figure 4.3). However, 48 hours later during 5-HT induced dose response contraction experiments (data not shown), this hyper-reactivity in the OVA exposed lungs was not obvious and % airway contraction had receded to similar levels to that in the non-sensitised airways. Excluding the potential for agonist pathway targeted differences in contractile response, the most obvious explanation was the influence of the inflammatory environment in retaining this increased airway responsiveness. Therefore to show AHR at its peak, the following experiments were designed to be achieved within 24 hours of mouse sacrifice and lung slice preparation; for each non-sensitised and matched OVA exposed model (acute, mid-chronic, chronic). The following results (figure 4.2) show % airway contraction from initial dilated airways to contraction to 10  $\mu$ M MCh perfusion for five minutes, for non-sensitised and OVA exposed mice. Corresponding representative images of airways are shown above.





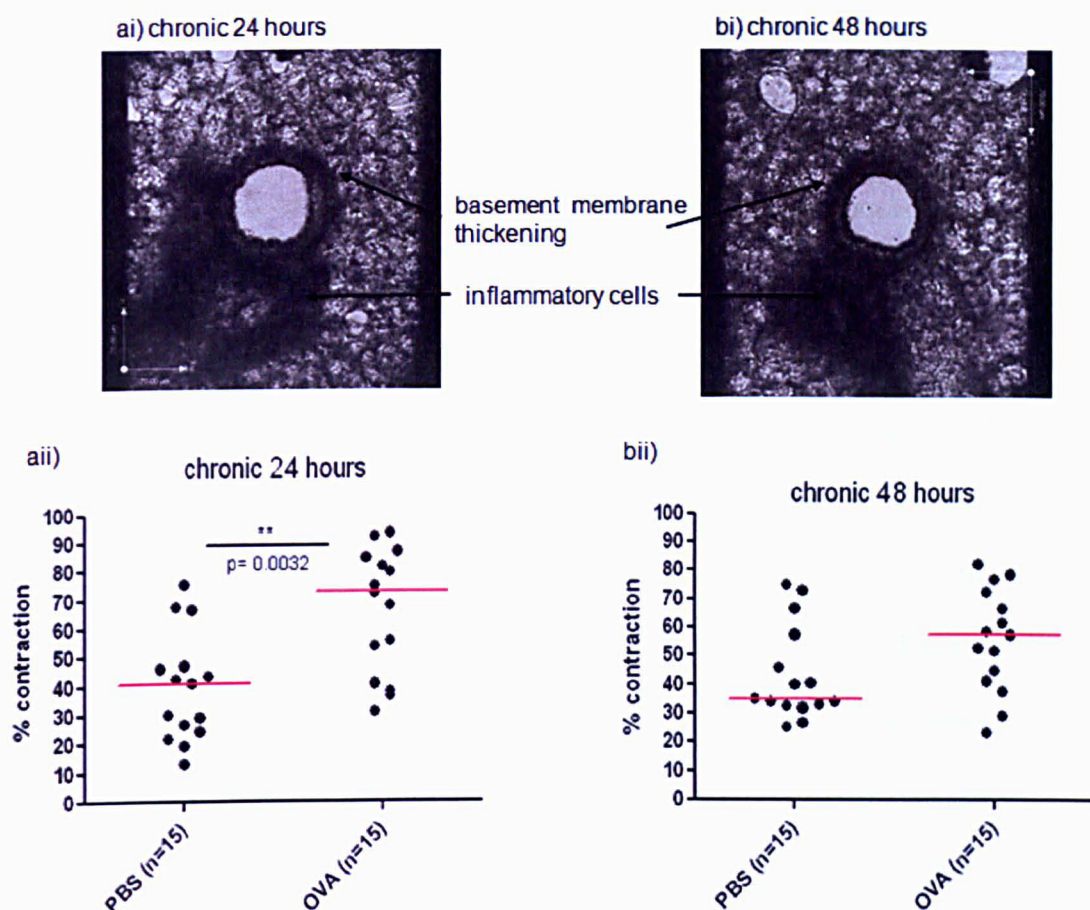
**Figure 4.2 Responsiveness of the airways to MCh is increased with repeated allergen exposure.** i) Above microscope images for a) acute, b) mid-chronic & c) chronic OVA allergen exposed mice, showing progressive inflammatory cell infiltration and increased airway wall thickness corresponding with repeated allergen exposure (130  $\mu$ m slices, 200x magnification, P = PBS control mouse, O = OVA sensitised mouse). ii) Airway contractions to a maximum concentration of 10  $\mu$ M MCh perfusion for five minutes in airways from mice that received either PBS or a) acute, b) mid-chronic & c) chronic OVA albumin allergen exposure. All experiments were performed within 24 hours of lung excision. 'n' equates to number of airways from individual mice, i.e n = 15 equates to 15 airways from 15 mice. % contraction was compared to the initial area of the fully relaxed airway. Statistical significance was assessed by the Mann Whitney test. Data are shown as scatter dot plot for individual mice, red lines indicate median. Airway responsiveness was significantly increased after mid-chronic and chronic sensitisation.  $P = 0.0168$  for mid-chronic sensitisation (median = 62.2% contraction) vs. matched PBS (median = 39.2%).  $P = 0.0032$  after OVA chronic sensitisation (median = 72.7% contraction) vs. matched PBS (median = 40.8%).



**Figure 4.3 OVA allergen exposure increases airway sensitivity to MCh.** Measurement of % airway contraction to an MCh dose response in BALB/c mice following OVA chronic sensitisation ( $n = \text{five mice}$ ), compared with time point matched PBS controls ( $n = \text{five mice}$ ). Experiments were performed within 24 hours of lung removal. % contraction is compared to the initial area of the fully relaxed airway.  $EC_{50}$  measurements were calculated by graphing the log of the agonist against the response for variable slope data. Data points represent median values, error bars show inter quartile range. The  $EC_{50}$  measurement to MCh in PBS airways was 206.2 nM (CI, 66.8 nM - 636.9 nM) compared with 84.7 nM (CI, 53.2 nM - 135.3 nM) in OVA airways. \*\* indicate statistical significance assessed using the Mann Whitney test for PBS vs. OVA sensitised groups for individual MCh concentrations. Differences in contractile response between PBS and OVA airways were significant at MCh concentrations above 0.5  $\mu\text{M}$  ( $p = 0.0079$ ).

The above data indicate enhanced airway responsiveness *in vitro* following *in vivo* sensitisation. I therefore proceeded to investigate how long these enhanced contractile responses persist in prolonged culture. Airway contraction in PBS and OVA chronic model sensitised airways was measured to 10  $\mu\text{M}$  MCh within 24, 48 and 72 hours after lung removal. Experiments revealed that while there was a significant difference in airway reactivity between PBS and sensitised airways within the first 24 hours; this significance was lost 48 hours after lung removal (figure 4.4). At three days post lung removal, magnitude of contraction between PBS and OVA airways were similar (data not shown).

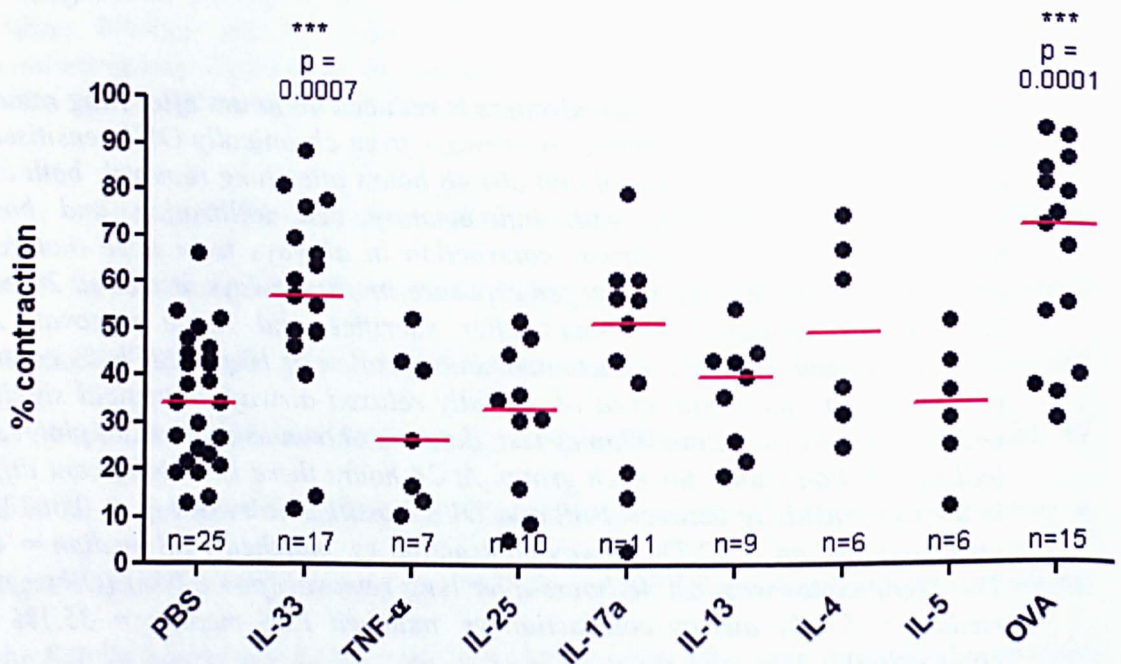




**Figure 4.4 Airway hyper-responsiveness is reduced 48 hours after lung removal.** i) representative bright field images of airways from chronically OVA sensitised mice ai) 24 hours after lung removal and aii) 48 hours after lung removal; both airways show airway remodelling with inflammatory cell infiltration and basement membrane thickening. ii) Airway contraction in airways from mice that received either PBS or chronic OVA allergen exposure in aii) airways measured 24 hours & bii) airways measured 48 hours after sacrifice and tissue removal. Airway contractions are shown to a maximum concentration of 10  $\mu$ M MCh. % contraction is compared to the initial area of the fully relaxed airway. Statistical significance was assessed by the Mann Whitney test, data are shown as scatter dot plot, red lines indicate median values for each group. At 24 hours there is a significant difference in airway sensitivity between PBS and OVA sensitised airways ( $p = 0.0032$ ) (OVA sensitised median = 72.7% airway contraction vs. matched PBS median = 40.8%). This significance was lost 48 hours after lung removal ( $p = 0.056$ ) (OVA sensitised median = 57.4% airway contraction vs. matched PBS median = 35.1% airway contraction).

### 4.3.2 IL-33 plays a key role in mediating airway hyper-responsiveness

Despite obvious airway structural remodelling in the sensitised airways, hyper-responsiveness was diminished 48 hours after lung removal (figure 4.4), suggesting the inflammatory environment may play a role in this enhanced airway response. The following experiment was designed to study the effects of pre-treatment with selected inflammatory mediators on magnitude of agonist induced airway contraction in the slices. Cytokines known to play a role in the  $T_H2$  inflammatory response were included (IL-4, IL-5, IL-9, IL-13, IL-17A, IL-25, TNF- $\alpha$  and IL-33), refer to table 4.1. Slices were cut from BALB/c wild type (WT) lung lobes within 24 hours of sacrifice and incubated overnight with 100 ng/ml of the chosen cytokine, contraction experiments were run the following day.

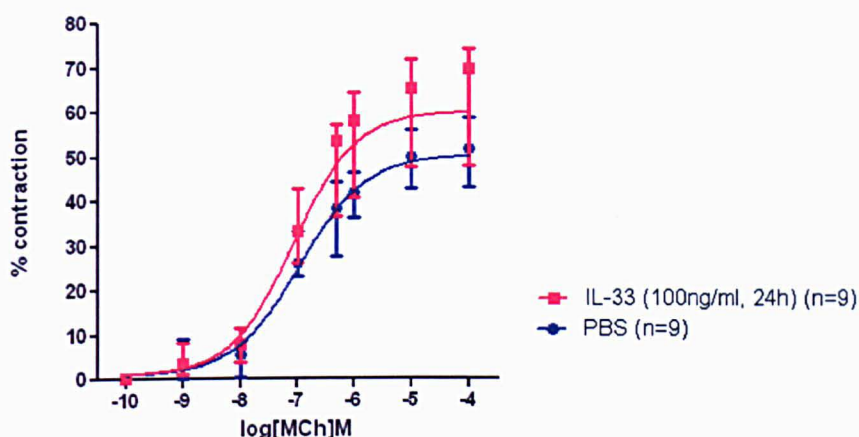


**Figure 4.5** Overnight exposure to IL-33 increases airway response to MCh. Results show % airway contraction to 10  $\mu$ M MCh following overnight treatment with a range of cytokines (100 ng/ml) in WT BALB/c mice; contraction in OVA chronic sensitised airways is also shown. % contraction was compared to the initial area of the fully relaxed airway. Statistical significance was assessed by the Mann Whitney test, comparing all data to PBS values. Data are shown as scatter plot for individual mice, lines indicate median values within each cytokine treatment group.



*PBS control median = 34.2% airway contraction. Of the cytokines selected, only IL-33 significantly increased airway responsiveness to MCh ( $p = 0.0007$ , median = 57.1% airway contraction). Data for OVA chronic sensitised mice is included for comparison ( $p = 0.0001$ , median = 72.7% airway contraction).*

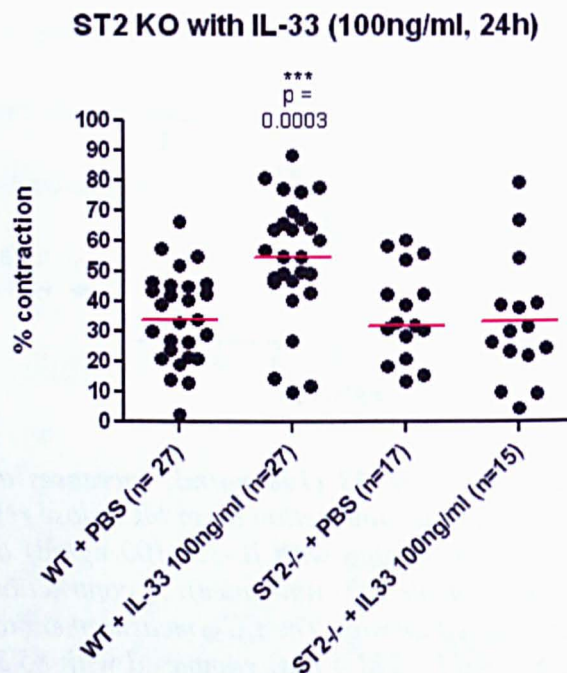
Given the increased maximal response to MCh seen following IL-33 exposure, I next investigated the effects of IL-33 on a range of doses to MCh (figure 4.6).



**Figure 4.6 Exposure to IL-33 (100 ng/ml) increases airway sensitivity to MCh.** Measurement of % airway contraction to an MCh dose response in WT BALB/c mice following overnight incubation with IL-33 (100 ng/ml) ( $n = \text{nine mice}$ ), compared with matched PBS controls ( $n = \text{nine mice}$ ). % contraction is compared to the initial area of the fully relaxed airway. The  $EC_{50}$  measurement to MCh in PBS airways was 108.6 nM (CI, 52.4 nM - 223.4 nM) compared with 85.3 nM (CI, 40.3 nM - 180.5 nM) in IL-33 exposed airways. Measurements were calculated using the log of the agonist vs. the response for variable sloped data. Data points indicate median, error bars indicate inter quartile range. Although a trend towards increased sensitivity and magnitude of contraction was seen the difference in the  $EC_{50}$  of the contractile response between PBS and IL-33 treated airways was not statistically significant.

IL-33, a recently named IL-1 family member (Baekkevold, Roussigne et al. 2003; Schmitz, Owyang et al. 2005; Cayrol and Girard 2009), signals via the IL-1 receptor related protein ST2 (Schmitz, Owyang et al. 2005), which may mediate production of cytokines and chemokines in mast cells (Allakhverdi, Smith et al.

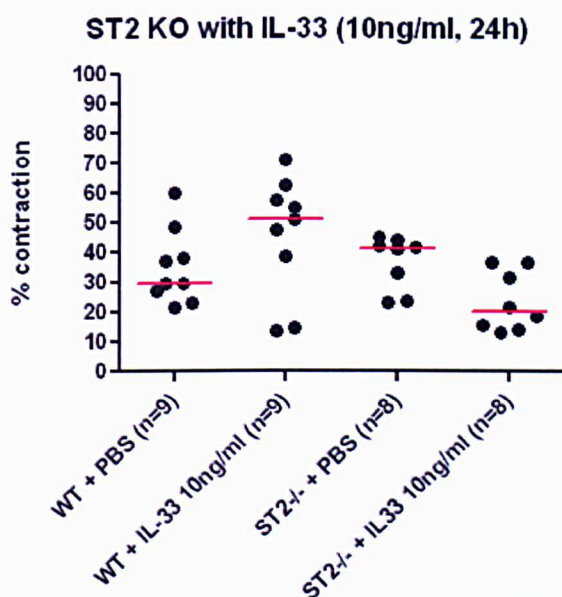
2007),  $T_H2$  lymphocytes (Smithgall, Comeau et al. 2008), NK cells (Bourgeois, Van et al. 2009), basophils and neutrophils (Cherry, Yoon et al. 2008). I therefore proceeded to investigate responses to IL-33 in an ST2 receptor (also named T1/ST2) KO mouse model, to show that this IL-33/ST2 receptor interaction was required for airway hyper-responsiveness. Experiments involved overnight incubation of lung slices with IL-33 (100 ng/ml) or PBS equivalent, airway contractions to 10  $\mu$ M MCh were measured the following day in the slices (figure 4.7a).



**Figure 4.7a IL-33 induced airway hyper-responsiveness is not found in the IL-33 receptor KO model.** Results show % airway contraction to 10  $\mu$ M MCh following overnight treatment with IL-33 (100 ng/ml) or equivalent PBS controls. 130  $\mu$ m thick slices were cut from either WT or ST2 receptor KO BALB/c mice. % contraction is compared to the initial area of the fully relaxed airway. Statistical significance was assessed by the Mann Whitney test, data points represent individual mice, red lines indicate median for each group. For WT + PBS exposed mice the median = 34.2% airway contraction, for ST2-/- + PBS exposed mice the median = 31.9% airway contraction. IL-33 exposure induced significantly increased airway sensitivity to MCh in WT mice (median = 55.2% airway contraction,  $p = 0.0003$ ). This increased responsiveness to IL-33 was lost in the ST2 receptor KO mice (median = 30.1% airway contraction).



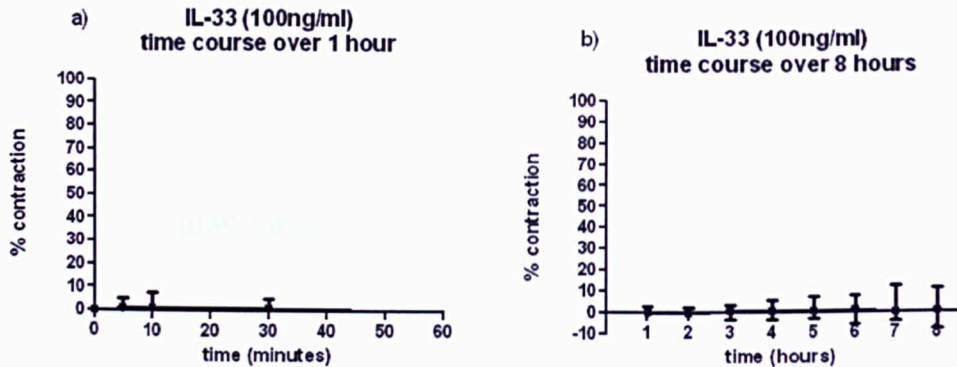
Lower degrees of inflammation would be expected to be associated with lower concentrations of IL-33; experiments were therefore repeated using 10 ng/ml of IL-33 (figure 4.7b). Although there was a trend towards increased responses in the WT mice, this no longer remained significant. As expected responses in the ST2 KO mice were similar to control.



**Figure 4.7b Airway hyper-responsiveness after IL-33 (10 ng/ml) exposure in WT and ST2 KO murine lung slices.** Results show % airway contraction to 10  $\mu$ M MCh following overnight treatment with IL-33 (10 ng/ml) or equivalent PBS controls. Slices were cut from either WT or ST2 receptor KO BALB/c mice. % contraction is compared to the initial area of the fully relaxed airway. Data points indicate values for individual mice, lines indicate medians for each group. Statistical significance was assessed by the Mann Whitney test. Although there is a trend towards increased contraction in WT mice after IL-33 exposure, this was no longer significant. No increase in responsiveness to IL-33 was seen in the ST2 receptor KO mice.

Previous studies have reported that such cytokines as IL-13 may directly induce contraction of ASM (Venkayya, Lam et al. 2002). To investigate whether IL-33 alone induced airway contraction directly, I exposed lung slices to IL-33 (100

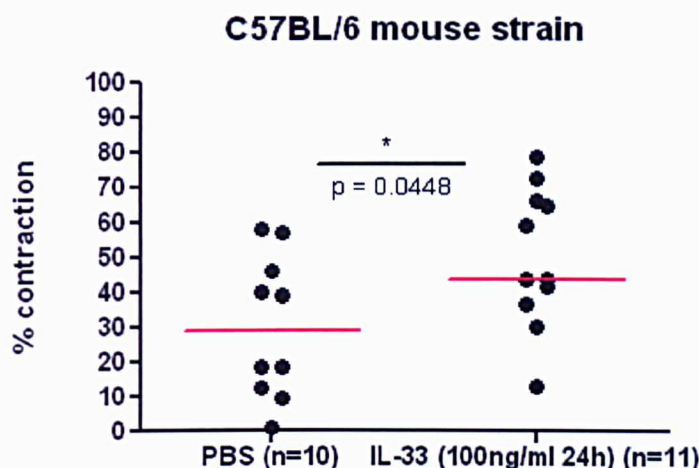
ng/ml) on a glass cover slip for image capture over 60 minute and eight hour time frames (figure 4.8).



**Figure 4.8 IL-33 alone does not induce immediate airway contraction.** Time course of % contraction for airways exposed to 100 ng/ml IL-33 over a) 60 minutes and b) eight hours. Lung slices were kept at 37°C, 5% CO<sub>2</sub> for the duration of the experiments and were removed from these conditions only to acquire data at the appropriate time points. Data show median values, error bars indicate inter quartile range; n = seven airways (three mice).

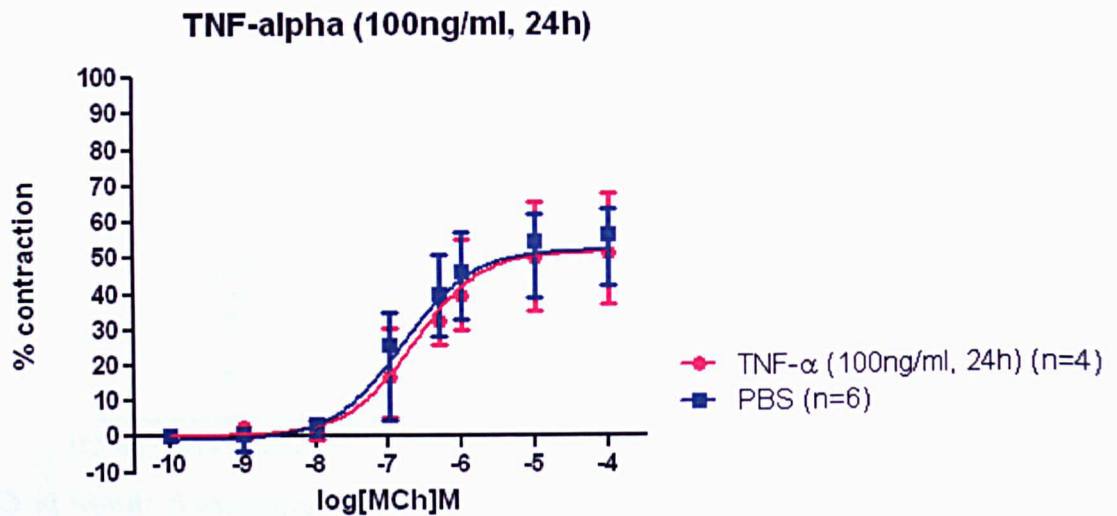
These results demonstrate that IL-33 by itself does not cause a contractile response of murine airways. All experiments shown so far in this chapter were conducted in BALB/c mice. As previous studies have indicated that BALB/c mice are genetically skewed towards a T<sub>H</sub>2 type response (Nishimura, Santa et al. 1997), experiments were repeated in C57BL/6 mice to see if this IL-33 induced AHR is strain specific (figure 4.9).





**Figure 4.9 Airway contraction increased by IL-33 exposure is shown in C57BL/6 mice.** Airway contractile response for C57BL/6 mouse lung slices ( $n =$  three mice) incubated overnight with IL-33 (100 ng/ml) (11 airways), or PBS (ten airways) prior to experiments. Statistical significance was assessed by the Mann Whitney test, data show values for individual airways, red lines indicate medians. IL-33 exposure lead to increased airway responsiveness to MCh in C57BL/6 mice ( $p = 0.0448$ , median = 43.7% airway contraction after IL-33 exposure vs. median = 28.8% airway contraction in PBS group).

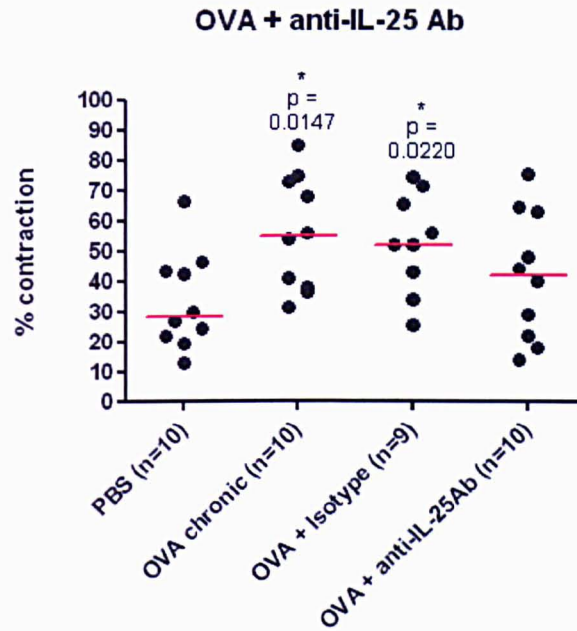
These results suggest that the effects of IL-33 are not strain specific. Whilst the initial results of experiments examining a range of cytokines (figure 4.5) did not show agonist induced enhanced responses after treatment, previous work has suggested enhanced  $\text{Ca}^{2+}$  responses in ASM preparations exposed to  $\text{TNF-}\alpha$ . One possible explanation for this difference might be altered sensitivity to agonists such as MCh without an enhanced maximum response. I therefore studied the response of murine lung slices to a range of concentrations of MCh after pre-exposure to  $\text{TNF-}\alpha$  (figure 4.10).



**Figure 4.10** Overnight exposure to TNF- $\alpha$  does not alter airway sensitivity to MCh. % airway contraction to MCh dose response following overnight treatment with TNF- $\alpha$  (100 ng/ml) ( $n =$  four mice) or equivalent PBS control ( $n =$  six mice). % contraction is compared to the initial area of the fully relaxed airway. No difference in airway sensitivity is shown between slices treated with TNF- $\alpha$  and PBS controls. The  $EC_{50}$  measurement to MCh in PBS airways was 150.7 nM (CI, 63.9 nM to 355.1 nM) compared with 213 nM (CI, 86.4 nM to 525 nM) in TNF- $\alpha$  exposed airways. Data was calculated by graphing the log of the agonist vs. the response for variable sloped data, data points are shown as median, error bars indicate inter quartile range.

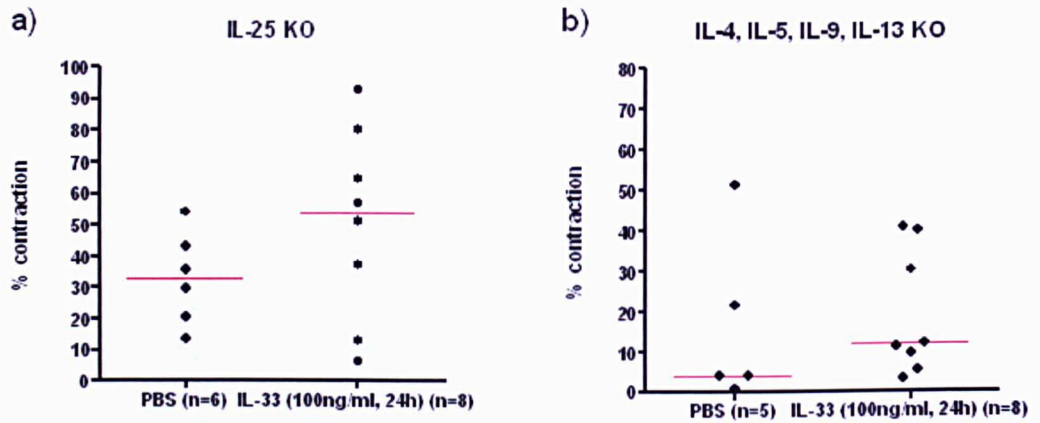
Cytokine blocking antibodies (Ab) were also considered to be administered on the OVA sensitised slices in the hopes of revealing a key mediator involved in AHR (figure 4.11).





**Figure 4.11 Overnight treatment with anti-IL-25 Ab reduces OVA exposure induced airway responsiveness to MCh.** % airway contraction to 10  $\mu$ M MCh in PBS and OVA chronic sensitised airways from BALB/c mouse lung slices, also showing contraction in OVA sensitised airways after overnight treatment with anti-IL-25 antibody (Ab) (10 ng/ml) and Ab isotype control. % contraction is compared to the initial area of the fully relaxed airway. Statistical significance was assessed by the Mann Whitney test, data represent individual mice, red lines indicate median values within each group. In comparison to PBS control airways (median = 28.3% airway contraction), OVA chronic sensitisation induced significant AHR to MCh ( $p = 0.0147$ , median = 54.7%), treatment with the anti-IL-25 Ab reduced this significance in the OVA sensitised airways (median = 41.7% contraction, not significant); the isotype control Ab did not reduce this significance (median = 51.9%,  $p = 0.022$ ).

Rapidly accumulating literature suggests the  $T_H2$  cytokines IL-13, IL-4, IL-5 and IL-9 play a role in the inflammatory response and AHR. One possibility is that the enhanced response seen to IL-33 is mediated by a range of the  $T_H2$  cytokines, but that exposure to a single cytokine such as IL-4 or IL-5 is insufficient to cause sensitisation. To address this issue I repeated IL-33 exposure of lung slices in slices from a  $T_H2$  KO mouse model, in which IL-4, IL-5, IL-9 and IL-13 are deleted (figure 4.12).



**Figure 4.12** % airway contraction to 10  $\mu$ M MCh following overnight treatment with IL-33 (100 ng/ml) or equivalent PBS control in airways from a) IL-25 KO and b) IL-4, -5, -9 & -13 KO mice. % contraction is compared to the initial area of the fully relaxed airway. Data points represent individual mice; lines indicate median values within each group. Using the Mann Whitney test to assess differences in contraction between PBS WT and KO treated airways, no significant difference in contraction was found. Although increased airway contraction is observed in IL-25-/- mice incubated with IL-33 (median = 53.9% airway contraction vs. median = 32.6% in PBS mice). Note: in the IL-4, IL-5, IL-9, IL-13 KO experiments, contractile responses were generally much smaller in WT mice.

## 4.4 DISCUSSION

In this chapter I aimed to explore mechanisms involved in the pathogenesis of airway inflammation and AHR; by applying the PCLS technique to a mouse model of allergen exposure induced airway disease. OVA sensitisation led to progressive structural remodelling and AHR to 10  $\mu$ M methacholine (MCh). However, this hyper-responsiveness was decreased 48 hours post lung removal. Based on initial results indicating that the inflammatory environment influences sensitivity of the airways (due to decline in AHR after 48 hours), lung slices were incubated with selected cytokines to investigate differences in contractile response. Of the inflammatory mediators assessed for lung slice incubation, IL-33 significantly increased AHR to MCh. In lung slices prepared from ST2 (IL-33 receptor) KO mice, IL-33 was unable to sensitise the contractile response. Results from this chapter suggest that the inflammatory environment promotes AHR and disassociates this airway sensitivity from structural remodelling. Importantly, this study implicates a role for IL-33 in mediating AHR in this murine model of allergic airway disease.

### 4.4.1 Structural remodelling and hyper-responsiveness is induced by repeated allergen exposure

In this study, an allergic inflammatory response was induced in murine lungs by sensitisation with ovalbumin. Mice were sacrificed after varying lengths of allergen challenge (refer to 2.1, acute, mid-chronic and chronic protocols). Progressive structural remodelling and inflammatory cell infiltration from blood vessels was seen with increasing allergen exposure. After chronic sensitisation,

significant airway remodelling was observed (figure 4.1, 4.2). These results are in keeping with previous studies which have noted correlations between length of allergen exposure and presence of extracellular matrix deposition (Leigh, Ellis et al. 2002), also suggesting that repeated allergen exposure may influence the degree of remodelling and hyper-responsiveness (Temelkovski, Hogan et al. 1998; Palmans, Kips et al. 2000; Trifilieff, El-Hashim et al. 2000).

Increasing lengths of allergen challenge corresponded to statistically significant increases in agonist induced AHR in lung slices between control and mid-chronic and chronic sensitised mice. After acute sensitisation, there was a trend towards an increase in airway contraction in OVA sensitised mouse lungs compared to PBS controls; however this increase was not significant. A dose response to MCh was conducted within 24 hours of sacrifice for the OVA chronically sensitised and PBS lung slices. Results showed a curve shift left and significant increase in magnitude of contraction at MCh concentrations above 0.5  $\mu\text{M}$ , reflective of increased airway sensitivity in the OVA sensitised airways. The  $\text{EC}_{50}$  measurement to MCh in PBS airways was 206.2 nM compared with 84.7 nM in OVA airways. Taken together these results show that the AHR which can be measured following *in vivo* exposure to agents such as MCh can be recapitulated *in vitro* in accurately prepared lung slices. No available  $\text{EC}_{50}$  values for PCLS models from untreated mice are present in the literature for comparison. A study using human PCLS (< 1.5 mm outer diameter) found an  $\text{EC}_{50}$  of 0.4  $\mu\text{M}$  to MCh in healthy airways (Wohlsen, Martin et al. 2003).



#### **4.4.2 Inflammatory environment influences hyper-responsiveness**

Using lung slices from mice exposed to chronic OVA sensitisation, airway contractions were induced by a maximum concentration of MCh within 24 and 48 hours of sacrifice. Hyper-responsiveness was observed in the OVA sensitised mice within 24 hours. However, by 48 hours, this reactivity was less significant, despite the airway structural remodelling present. This experiment was continued for 72 hours after sacrifice and again the increased reactivity initially measured in the OVA sensitised mice appeared to decline further, to similar levels of contractile response measured in the control mice. This suggests that the inflammatory environment influences sensitivity of the airways and dissociates AHR from structural remodelling as inspection of the airways at 72 hours, as expected did not show any reduction in the extent of airway remodelling. Using a different protocol, a previous paper measuring constriction six weeks after challenge noted no difference between sensitised and control mice (Chew, Hirota et al. 2008), supporting these findings that persistent inflammatory mediators are required for sensitisation. This prompted further investigation of inflammatory mediator candidates.

#### **4.4.3 Manipulation of lung slice inflammatory environment suggests IL-33 plays a role in AHR**

Animal models of airway inflammation are progressively implicating inflammatory mediators which may be involved in initiating this process. Previous studies have shown that specific allergens may divert the immune response towards a  $T_H2$  phenotype (Schulz, Sewell et al. 1998) with induction of cytokines (Seder, Paul

et al. 1992), although the precise mechanisms by which  $T_H2$  cytokines mediate inflammatory infiltration and AHR are still unclear.

Experiments were designed to explore the influence of overnight cytokine incubation of the naïve mouse slice on their contractile behaviour. Key inflammatory mediator candidates chosen were i) IL-4 for its possible role in initial differentiation of the  $T_H2$  cells (Brusselle, Kips et al. 1994; Coyle, Le Gros et al. 1995; O'Garra and Arai 2000; Murphy and Reiner 2002). ii) IL-5 for its link to eosinophil infiltration and possible fibrosis (Robinson, Hamid et al. 1992; Koike and Takatsu 1994; Takatsu, Takaki et al. 1994; Akutsu, Kojima et al. 1995; Foster, Hogan et al. 1996; Lee, McGarry et al. 1997; Leckie, ten Brinke et al. 2000; Tanaka, Komai et al. 2004; Rothenberg and Hogan 2006). iii) IL-9 which has been suggested to have a genetic link to AHR and mast cell development (Nicolaidis, Holroyd et al. 1997; Shimbara, Christodoulopoulos et al. 2000; Townsend, Fallon et al. 2000). iv) IL-13, which is shown to influence epithelial and smooth muscle cell inflammation (Walter, McIntire et al. 2001). v) IL-17A for its link between activation of T cells and recruitment of neutrophils (Jatakanon, Uasuf et al. 1999; Fort, Cheung et al. 2001; Linden 2001; Moseley, Haudenschild et al. 2003; Kawaguchi, Adachi et al. 2004; Weaver, Harrington et al. 2006). vi) IL-25 for its possible role in amplifying the  $T_H2$  response (Jatakanon, Uasuf et al. 1999; Fort, Cheung et al. 2001; Pan, French et al. 2001; Ikeda, Nakajima et al. 2003; Babu, Davies et al. 2004; Tamachi, Maezawa et al. 2006). vii) TNF- $\alpha$  for its induction of cytokines and chemokines involved in asthma (Kips, Tavernier et al. 1992; Thomas, Yates et al. 1995; Keatings, Collins et al. 1996; Babu, Davies et al. 2004; Berry, Hargadon et al. 2006). viii) Finally, IL-33 as IL-33/ST2 interaction may play a role in induction of airway inflammation through mast cell mediated inflammation (Lohning, Stroehmann et al. 1998;

Trajkovic, Sweet et al. 2004; Schmitz, Owyang et al. 2005; Allakhverdi, Smith et al. 2007) (refer to table 4.1).

Of the inflammatory mediating candidates chosen, overnight incubation with IL-33 (100 ng/ml) was shown to significantly increase the contraction of the mouse peripheral airways (figure 4.5) to contraction levels comparable to the chronic OVA sensitised mice, where as none of the other mediators recapitulated the effect of OVA.

#### **4.4.4 IL-33 influences airway hyper-responsiveness**

Further attempts to explore the role of IL-33 in influencing mechanisms involved in AHR involved an IL-33 receptor (ST2) KO mouse model. Results in figure 4.7a, show that wild type BALBc mice incubated with IL-33 (100 ng/ml) overnight, showed significantly increased contractile responses in comparison to controls ( $p = 0.003$ ). However, in the IL-33 receptor (ST2) knockout mice, no hyper-responsiveness was observed after incubation with IL-33.

IL-33's ability to stimulate contraction directly was considered, given that such cytokines as IL-13 have been reported to directly act on ASM (Venkayya, Lam et al. 2002). To show that IL-33 itself did not induce a contraction, a time course was set up with IL-33 incubation of the slices (figure 4.8). No contraction was observed within an hour, or over an eight hour period with exposure to 100ng/ml IL-33, indicating that IL-33 does not act directly on the ASM and cannot modulate contraction without an agonist present.

Previous experiments in this chapter involved the use of BALB/c mice, selected for sensitised models due to their preferential  $T_H2$  response (Nishimura, Santa et al. 1997). However, it was necessary to show that IL-33's effects on AHR were not strain selective; therefore these experiments were repeated in C57BL/6 mice. Again IL-33 (100 ng/ml) was able to sensitise airways inducing an increased response to MCh in comparison to controls.

From previous literature there is evidence to suggest TNF- $\alpha$  may play a role in hyper-reactivity. Treatment with the TNF- $\alpha$  antagonist 'etanercept' was shown to reduce airway hyper-reactivity in patients with refractory asthma (Berry, Hargadon et al. 2006). Using inhaled recombinant human TNF- $\alpha$  on normal patients, hyper-responsiveness and increased sputum neutrophils were noted (Thomas, Yates et al. 1995). Experiments in this chapter, designed to study the effects of recombinant mouse TNF- $\alpha$  on AHR, showed that slice incubation with TNF- $\alpha$  100 ng/ml did not significantly increase airway contraction to a maximum concentration of MCh, 10  $\mu$ M (figure 4.5). Additionally, no differences in airway sensitivity between TNF- $\alpha$  exposed and PBS airways were seen with a MCh dose response (0.001 – 100  $\mu$ M) (figure 4.10).

The activities of TNF- $\alpha$  are mediated by two distinct receptors, p55 (TNFR1) and p75 (TNFR2). A study in mouse tracheal rings treated with TNF- $\alpha$ , demonstrated that carbachol induced isometric force was significantly increased after treatment compared with controls. However, this enhanced response was abolished in p55R deficient mice and in control rings treated with a TNF- $\alpha$  mutant that only activates the p75R (Chen et al. 2003). A study by Kanehiro and colleagues, 1992, sensitising p55 and p75 KO mice with ovalbumin revealed that p75 deficient mice

developed AHR with similar levels to control mice. However, sensitised p55 KO mice failed to develop AHR (Kanehiro, Lahn et al. 2002). Furthermore in p55 deficient mice IL-5, IL-13, IFN- $\gamma$  and IL-12 were significantly reduced compared to controls, with greater reduction than in p75 deficient mice; suggesting a dominant role for the p55 receptor pathway in T<sub>H</sub>1 and T<sub>H</sub>2 cytokine production and eosinophil recruitment to the lung. In human, TNF- $\alpha$  also induces trachea smooth muscle cell proliferation by activation of the p55 receptor (Amrani, Aubier et al. 1996). Given the independent cell expression (Ryffel and Mihatsch 1993) and lack of homology of the intracellular domains (Dembic, Loetscher et al. 1990), these receptors are likely to have different functions. p75 has greater specificity and affinity for TNF- $\alpha$  interactions than does p55 (Medvedev, Espevik et al. 1996), with the suggestion that p75 functions in transmitting activating signals for apoptosis (Bazzoni and Beutler 1996; Hsu, Shu et al. 1996) and also to bind TNF- $\alpha$  and deliver it to the p55 receptor (Tartaglia and Goeddel 1992). Most interestingly, the murine p55 receptor binds both murine and human TNF- $\alpha$ , however, the murine p75 receptor only binds murine TNF- $\alpha$  and not the human protein (Lewis, Tartaglia et al. 1991). Taking into account these findings, my results suggesting that recombinant murine TNF- $\alpha$  exposure did not sensitise airways may actually be reflecting conflicting actions of the murine p55 and p75 receptors. Using recombinant human TNF- $\alpha$  on the mouse lung slices, with an affinity for the p55 receptor only, I would expect increased sensitivity of the mouse airways. Furthermore the specific differences in binding affinity of the mouse p55 and p75 receptors present an opportunity for dissecting the distinct receptor roles in the inflammatory response and AHR.

The work summarised above had established that IL-33 was an upstream mediator of AHR; however, the downstream targets responsible for altered contraction in the ASM remained unclear. In an attempt to unravel the major players involved, experiments involving blocking Ab and KO mice were conducted. IL-25 was considered based on previous literature documenting its role in inducing IL-4, IL-5 and IL-13 production from undefined non-T, non-B cells, resulting in blood eosinophilia, increased serum IgE levels and pathological changes in lung and other tissues (Tamachi, Maezawa et al. 2006). Experiments involved treating OVA chronically sensitised lung slices with an anti-IL-25 Ab, overnight. OVA chronic sensitised airways showed significant AHR to MCh. Treatment with the anti-IL-25 Ab reduced this significance in the OVA sensitised airways; the isotype control Ab did not reduce this significance. These results indicate a potential role for IL-25 in the inflammatory pathway leading to altered airway sensitivity.

These experiments were trialled on a larger scale using KO mice: for IL-25<sup>-/-</sup> and for the combination: IL-4<sup>-/-</sup>, IL-5<sup>-/-</sup>, IL-9<sup>-/-</sup> and IL-13<sup>-/-</sup> (figure 4.3.2.8). These KO lung slices were incubated over night with IL-33 for contractile experiments to MCh. In IL-25 KO lung slices treated with IL-33, airway reactivity was increased but not significant compared to PBS treated controls. In the IL-4, IL-5, IL-9 and IL-13 KO lung slices both PBS control and IL-33 treated airways gave similar yet small contractions (< 30%). Without a reliable contraction in control mice, results could not be confidently interpreted. However, a general trend suggested that the IL-4, IL-5, IL-9 and IL-13 KO airways showed a reduced response and that exposure to IL-33 by itself did not reinstate this increased reactivity. These experiments should have been repeated, however, with the availability of tissue and difficulty in breeding such KO mice, the opportunity only arose once. Despite the disappointing outcome for



this experiment, use of such KO mice and blocking Ab provides an exciting approach to further unravelling the mediators of AHR in this lung slice model.

#### **4.4.5 Does IL-33 have relevance for the treatment of human asthma?**

IL-33 has been reported to act intracellularly as a transcription factor and extracellularly as a NF- $\kappa$ B inducing cytokine (Carriere, Roussel et al. 2007). The membrane bound form of the mouse IL-33 receptor (T1/ST2) forms a complex with IL-33 to activate NF- $\kappa$ B and MAP kinases and drive production of T<sub>H</sub>2 cytokines (IL-4, IL-5 and IL-13) (Schmitz, Owyang et al. 2005). The detailed mechanisms are discussed further in Chapter Five. In murine models administration of exogenous IL-33 has produced splenomegaly, increased mucous production and hypertrophy in the respiratory and digestive tract (Schmitz, Owyang et al. 2005), IL-33's role has also been suggested in the immune response in the CNS (Hudson, Christophi et al. 2008). Previous studies have shown that T1/ST2 is preferentially expressed on murine T<sub>H</sub>2 cells (Coyle, Lloyd et al. 1999). Antigen specific T<sub>H</sub>2 cytokine responses and effector function were impaired in T1/ST2 deficient mice (Townsend, Fallon et al. 2000). Administration of a T1/ST2 mAb or a T1/ST2 fusion protein reduced the induction of a T<sub>H</sub>2 immune response in lung mucosa (Lohning, Stroehmann et al. 1998). Studies in a murine model of airway inflammation showed induced expression of IL-33 and the soluble and membrane bound forms of ST2. The soluble form of ST2 is suggested to act as an inhibitor of IL-33/ST2 signalling as its addition reportedly blocked the induction of T<sub>H</sub>2 cytokines (IL-4, IL-5, IL-13) from IL-33 stimulated splenocytes and suppressed activation of NF- $\kappa$ B (Hayakawa, Hayakawa et al. 2007). Rapidly accumulating evidence suggests a critical role for IL-33 in

allergic airway inflammation in animal models; however, the clinical relevance for human airways remains unclear.

Only recently studies have explored expression of IL-33 in human tissue and its role in the  $T_H2$  response in diseased states. Use of cDNA libraries suggests human IL-33 is predominantly expressed by structural cells including fibroblasts and endothelial cells (Schmitz, Owyang et al. 2005; Carriere, Roussel et al. 2007). Recently IL-33 expression has been shown at the mRNA and protein level on ASM (Prefontaine, Lajoie-Kadoch et al. 2009). IL-33 expression was shown to be increased in bronchial biopsies from patients with asthma in comparison to controls.

These data suggest IL-33 plays a role in the inflammatory response and AHR in animal models, with potential translation to the allergic inflammatory response in human. Future studies will involve contractile experiments after treatment of naïve mouse lung slices with bronchoalveolar lavage fluid taken from OVA sensitised mice and supernatants taken from OVA sensitised mouse lung slices. These experiments will attempt to demonstrate the essential requirement of the cytokines identified in these studies; that are present in the inflammatory environment to initiate and maintain AHR. The next chapter explores the mechanisms involved in IL-33 mediated AHR with particular emphasis on the downstream inflammatory mediators and cells targeted by IL-33.

## **CHAPTER FIVE RESULTS**

### **MECHANISMS OF IL-33 MEDIATED** **AIRWAY HYPER-RESPONSIVENESS**

## 5. CHAPTER FIVE

### 5.1 INTRODUCTION

In the previous chapter a potential key role for IL-33 in mediating enhanced airway contractile responses to MCh was identified using an OVA sensitised mouse model. OVA sensitisation resulted in structural changes, increased eosinophil infiltration around the airways and increased reactivity to MCh in *ex vivo* lung slices. This hyper-responsiveness was decreased 48 hours after lung removal despite airway remodelling. Of the cytokines chosen to study which might influence airway sensitivity, IL-33 significantly increased AHR to MCh in naïve lung slices. This increased airway contractile response induced by IL-33 exposure in naïve, WT BALB/c and C57BL/6 mouse lung slices suggests that IL-33 was able to produce AHR in the absence of previous sensitisation *in vivo*. As demonstrated using the ST2 receptor KO mice, this AHR pathway requires an interaction between IL-33 and the ST2 membrane receptor. Most significantly, these results reflect the influence of the inflammatory environment on ASM function, independent of structural remodelling.

Although IL-33 is a recently discovered IL-1 family member (Baekkevold, Roussigne et al. 2003; Schmitz, Owyang et al. 2005; Cayrol and Girard 2009), rapidly accumulating studies have supported the role for IL-33 in airway inflammation; further attempts are now being made to unravel the mechanisms involved. Before designing the experiments for this chapter, previous literature was reviewed to summarise the known role of IL-33 in various cell types (table 5.1), to identify common pathways in inflammatory cells and structural cells. Published data for expression profiling of IL-33 and the ST2 full length transmembrane receptor (ST2L) are summarised in table 5.2.

In this chapter the regulation of the IL-33/ST2 receptor axis was further explored to determine if increased IL-33 and ST2 receptor expression were shown in sensitised lung slices. This regulatory axis was also investigated in the human system to determine whether IL-33 targets the ASM directly.

In addition, given the possibility that enhanced contractile responses of ASM could be due to altered  $\text{Ca}^{2+}$  handling (as has previously been demonstrated by IL-13 induced upregulation of the RhoA protein, involved in  $\text{Ca}^{2+}$  sensitivity (Chiba, Nakazawa et al. 2009)); I also explored the possible regulation by IL-33 of key proteins involved in  $\text{Ca}^{2+}$  homeostasis, namely STIM1 and Orai1. Dr S. Peel (Therapeutics and Molecular Medicine Department) had previously shown that STIM1 and Orai1 play a role in SOCC activation following store depletion in human ASM (Peel, Liu et al. 2008). The depletion of  $\text{Ca}^{2+}$  results in dissociation of  $\text{Ca}^{2+}$  from STIM1, which causes STIM1 to relocate within the ER to areas near Orai1 channels located in the plasma membrane. STIM1 is then proposed to activate  $\text{Ca}^{2+}$  selective Orai1 channels (Smyth, Dehaven et al. 2006; Putney 2007). IL-33 mediated regulation of STIM1-Orai1 activity was considered as a potential pathway for increased ASM cell  $\text{Ca}^{2+}$  entry and resulting increased airway contraction; therefore experiments were designed to investigate differences in expression of STIM1 and Orai1 between OVA sensitised and matched non-sensitised mouse lung tissue.

**Table 5.1 Evidence to support the role of IL-33 in selected cell types promoting the airway inflammatory response.**

Cell type	Evidence for the role of IL-33 in inflammation
Cardiac muscle	Expression in cardiac myocytes, soluble ST2 release in response to myocardial infarction (Weinberg, Shimpo et al. 2002).
ASM	Higher levels of IL-33 were detected in biopsies from asthmatic patients compared to controls. IL-33 was shown to be expressed at both protein and mRNA levels in ASM cells (Prefontaine, Lajoie-Kadoch et al. 2009).
Endothelial	IL-33 was initially discovered as a nuclear factor expressed in high endothelial venules (NF-HEV), specialised blood vessels which mediate lymphocyte recruitment into lymphoid organs. This spawned theories that IL-33 may act as both a cytokine and nuclear factor, similarly to IL-1 $\alpha$ and chromatin-associated cytokine HMGB1. IL-33 is suggested to play a role as an endogenous danger signal to alert the immune system after endothelial or epithelial cell damage (Moussion, Ortega et al. 2008). IL-33 is constitutively expressed in large and small blood vessels in most tissues (> 50) and is expressed in endothelial cells from lymphoid tissues, rheumatoid arthritis and Crohn's disease. IL-33 was also expressed in the nucleus of endothelial cells in specific human tumours, suggesting IL-33 is a marker of tumour blood vessels. These data suggest that IL-33 is a nuclear marker of the endothelium in normal and chronically inflamed human tissues (Moussion, Ortega et al. 2008).
Epithelial	Using immunostaining for IL-33 in endobronchial tissue sections from patients with mild, severe asthma and controls; the bronchial epithelium of patients with severe asthma showed elevated IL-33 levels over those with mild asthma and controls (Prefontaine, Nadigel et al. 2009). In a recent study ST2 showed expression in epithelial and endothelial cells (not fibroblasts or smooth muscle), in this study IL-33 mediated the production of IL-6, IL-8 and MCP-1 from epithelial cells and microvascular endothelial cells (Orihara, Yagami et al. 2009).
Mast cells	In naïve murine bone marrow-derived, cultured mast cells IL-33 promoted IL-13 and IL-6 production. IL-33 promoted cytokine production via a MYD88-dependent pathway (Ho, Ohno et al. 2007). Administration of IL-33 to WT mice exacerbated collagen-induced arthritis and increased production of proinflammatory cytokines and anti-collagen Ab. Mast cells expressed high levels of ST2L and after IL-33 stimulation produced inflammatory cytokines and chemokines (IL-5, IL-13 but not IL-4) (Xu, Jiang et al. 2008).
Macrophage	IL-33 changed the phenotype of alveolar macrophages toward an alternatively activated macrophage (AAM) phenotype that expressed the mannose receptor, IL-4R $\alpha$ and produced IL-13 dependent high levels of CCL24 and CCL17. IL-33 increased IL-13 induced polarization of alveolar and bone marrow derived macrophages towards an AAM phenotype, through IL-13/ IL-4R $\alpha$ signalling for inducing expression of ST2L. Increased expression of IL-33 in lung epithelial cells of asthmatic patients



	compared to healthy controls was also shown (Kurowska-Stolarska, Stolarski et al. 2009).
Eosinophil	IL-33 increased eosinophil survival and increased cell surface expression of the intercellular adhesion molecule (ICAM1) on eosinophils (Chow, Wong et al.). IL-33-mediated increased survival of adhesion molecules and release of cytokines and chemokines were differentially regulated through activation of the nuclear factor (NF)- $\kappa$ B, p38 mitogen activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathway. Aside from basophils, eosinophils are the only other leukocyte-type directly activated by IL-33, shown by screening of p38 activation in peripheral blood cells (Pecaric-Petkovic, Didichenko et al. 2009).
Basophil	Using mouse bone marrow derived cultured basophils, IL-18 and IL-33 stimulation lead to production of IL-4, IL-13. IL-18 and IL-33 activation of T <sub>H</sub> 2 cytokine production occurs through MYD88 and p38 $\alpha$ signalling proteins (Kroeger, Sullivan et al. 2009). Human basophils express IL-18 receptors, ST2 surface expression is inducible by IL-3. IL-33 promotes IL-4, IL-13 and IL-8 secretion with IL-3 and/or Fc $\epsilon$ RI-activation and enhances Fc $\epsilon$ RI-induced mediator release. IL-33 activates NF- $\kappa$ B and shows a preference for p38 MAP kinase, while IL-3 acts through JAK/STAT and preferentially activates ERK (Pecaric-Petkovic, Didichenko et al. 2009).

**Table 5.2 Published data on the expression of IL-33 and the ST2L (membrane bound) receptor in a range of cell types in human tissues.**

Expression	IL-33	ST2L	Reference
ASM cells	RNA		(Prefontaine, Lajoie-Kadoch et al. 2009), this study
Endothelial cells (vascular, lung)	RNA, protein	RNA	(Moussion, Ortega et al. 2008; Prefontaine, Lajoie-Kadoch et al. 2009)
Epithelial cells (lung)	RNA, protein	RNA	(Prefontaine, Nadigel et al. ; Schmitz, Owyang et al. 2005; Moussion, Ortega et al. 2008), this study
Mast cells		RNA	(Allakhverdi, Smith et al. 2007), this study
Macrophages	RNA		(Schmitz, Owyang et al. 2005)
Fibroblasts	Protein	RNA	(Moussion, Ortega et al. 2008)
Dendritic cells	RNA		(Schmitz, Owyang et al. 2005; Turnquist, Sumpter et al. 2008)
T lymphocytes		RNA, protein	(Oshikawa, Yanagisawa et al. 2002)
Eosinophils, Basophils		Protein	(Chow, Wong et al. ; Pecaric-Petkovic, Didichenko et al. 2009; Valent 2009)

### 5.1.1 AIMS

The aims of the work described in this chapter were i) to investigate regulation of expression of IL-33 and ST2 soluble and membrane bound receptor mRNA in OVA sensitised mouse lung tissue using quantitative PCR and ii) to examine expression in these samples of the key proteins STIM1 and Orai1, involved in ASM  $\text{Ca}^{2+}$  homeostasis and store operated  $\text{Ca}^{2+}$  entry.

## **5.2 METHODS**

All relevant methods for cell culture, RNA isolation, cDNA synthesis, protocols for quantitative PCR, Affymetrix microarray data and analysis are described in section 2.3.

### **5.2.1 Statistical analysis**

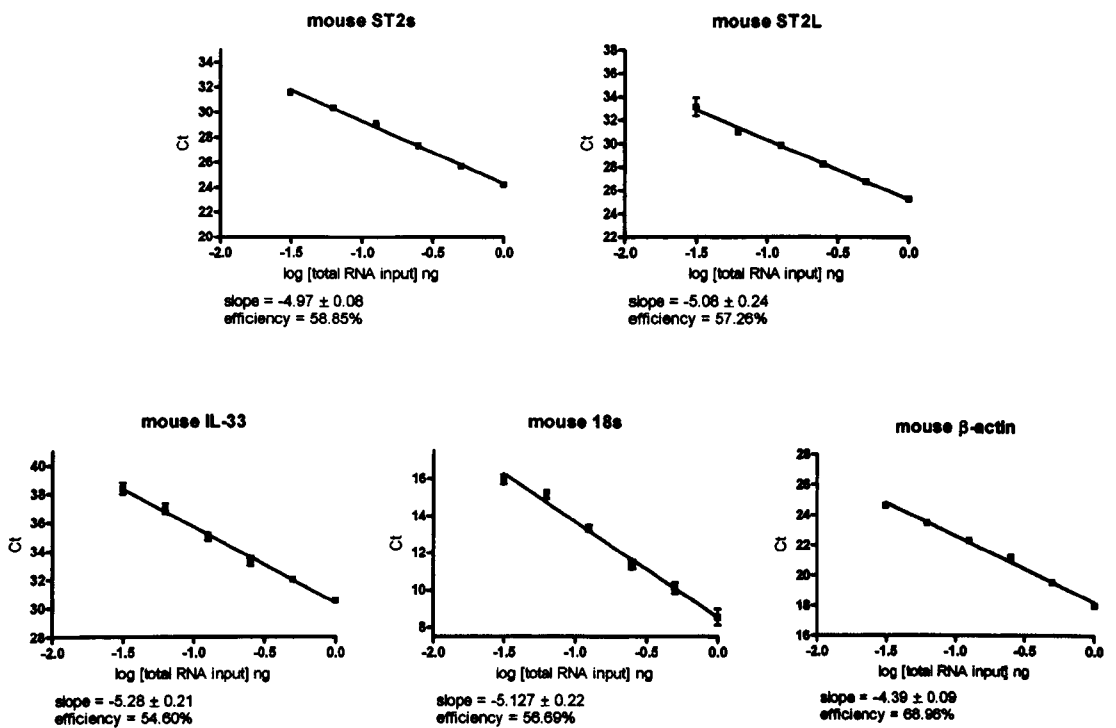
Using PRISM software Graphpad 5.02, statistical significance was assed by the Mann Whitney test, which ranked the data and compared medians. For all scatter plots, each dot represents an individual mouse, red lines indicate median values ('n' refers to number of mice).

### 5.3 RESULTS

Quantitative PCR (Q-PCR) experiments were designed to investigate altered expression of STIM1, Orai1, IL-33 and the ST2 soluble (ST2s) and membrane bound (ST2L) receptors in OVA sensitised mouse lung tissue. Initial experiments were performed to examine the efficiency of Q-PCR reactions for the target genes. Figure 5.1 shows standard curves for murine IL-33, ST2L, ST2s and the potential housekeeping genes, 18S and  $\beta$ -actin. Initial results suggested little difference in terms of performance of murine 18S and  $\beta$ -actin as housekeeping genes and the results for murine experiments shown in this chapter were all standardised to  $\beta$ -actin. Figure 5.4 shows standard curves for Q-PCR reactions for murine STIM1, Orai1 and  $\beta$ -actin. Primer efficiencies were determined by plotting a cDNA standard curve. Although primer efficiencies were not 100%, they were all similar in value to each other; therefore for comparative analysis the  $2^{-\Delta\Delta C_t}$  equation was used (see methods, 2.3.6). For results shown, reactions for PBS and OVA cDNA samples were repeated in triplicate for all genes examined. The 'n' number equates to the number of individual mice used, individual scatter points represent the mean of the three values found for individual cDNA samples, from individual mice. Data were analysed using the Mann Whitney test, with mRNA expression measured relative to the  $\beta$ -actin house keeping gene.

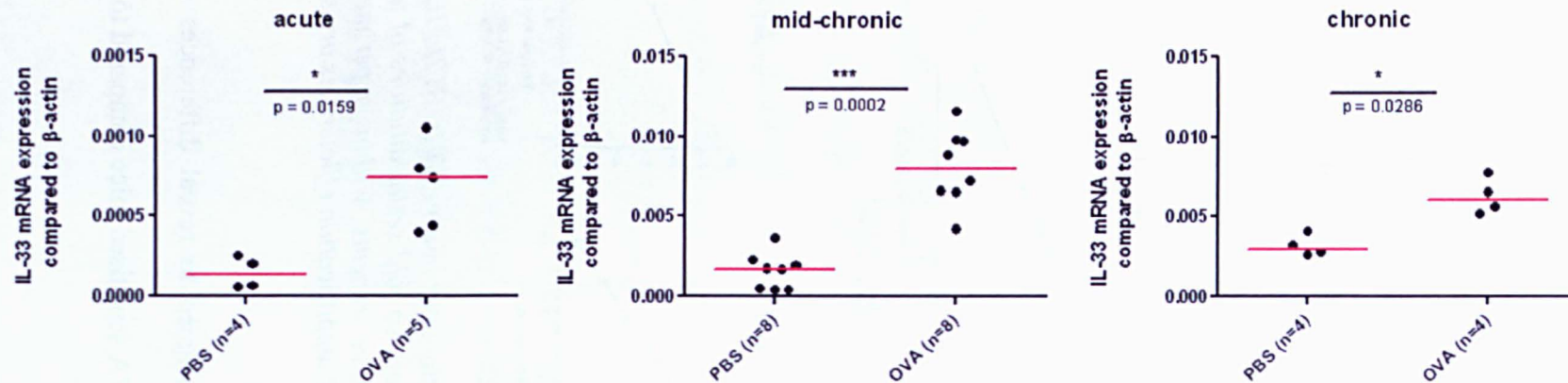
### 5.3.1 Increased expression of IL-33, ST2s and ST2L in ovalbumin sensitised lung tissue

Prior to Q-PCR experiments targeting IL-33, ST2 soluble (ST2s) and ST2 membrane bound (ST2L) primer efficiencies were calculated for the assays (figure 5.1) from cDNA standard curves using cultured naïve WT mouse trachea smooth muscle cells (see methods, 2.2.1).



**Figure 5.1** Standard curves for Q-PCR reactions for murine ST2s, ST2L, IL-33, 18S and β-actin. In each case the amplification of two fold serial dilutions of naïve WT mouse trachea smooth muscle cDNA (three repeats within experiment) was performed. The slope of each graph and PCR amplification efficiencies are shown.

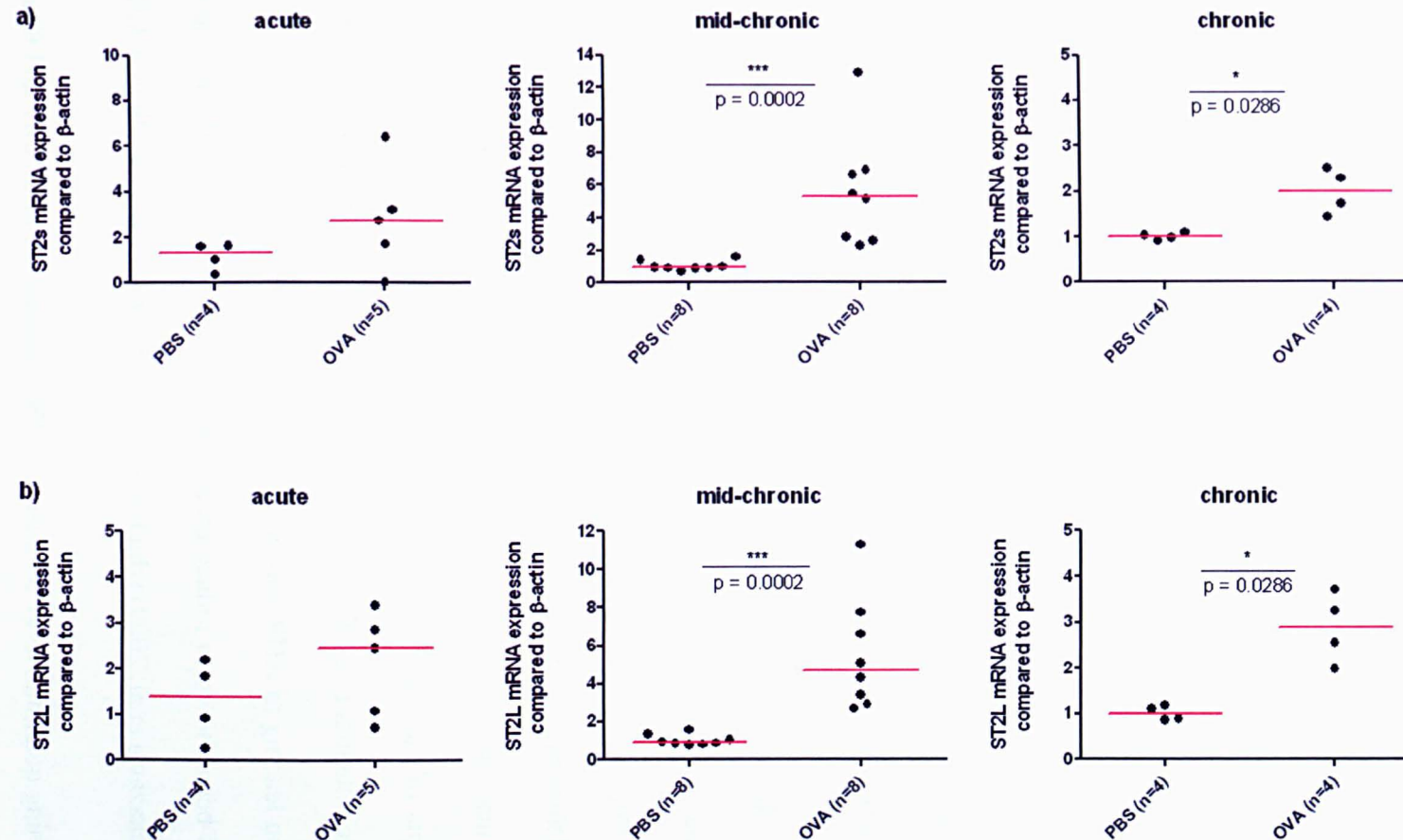
Initial Q-PCR experiments were designed to reveal differences in IL-33 expression in whole lung tissue from the OVA sensitised mice compared to controls (figure 5.2).



**Figure 5.2 IL-33 mRNA expression is increased in OVA sensitised lung tissue.** Quantitative PCR results showing mRNA expression of IL-33 in acute, mid-chronic and chronic OVA sensitised BALB/c mouse lung tissue compared with PBS controls. Data were analysed using the Mann Whitney test, with IL-33 mRNA expression measured relative to the  $\beta$ -actin house keeping gene. Results showed significantly increased expression of IL-33 in all OVA sensitised models (acute, mid-chronic, chronic) compared to matched non-sensitised mice.

Given this up regulated expression of IL-33 in the sensitised lung tissue, further Q-PCR experiments were designed to investigate whether ST2 receptor transcription was also increased in these samples. Results showed increased expression of the alternatively spliced secreted soluble form of the ST2 receptor (ST2s) and the full length transmembrane form (ST2L) in mid-chronic and chronic sensitised models (figure 5.3).





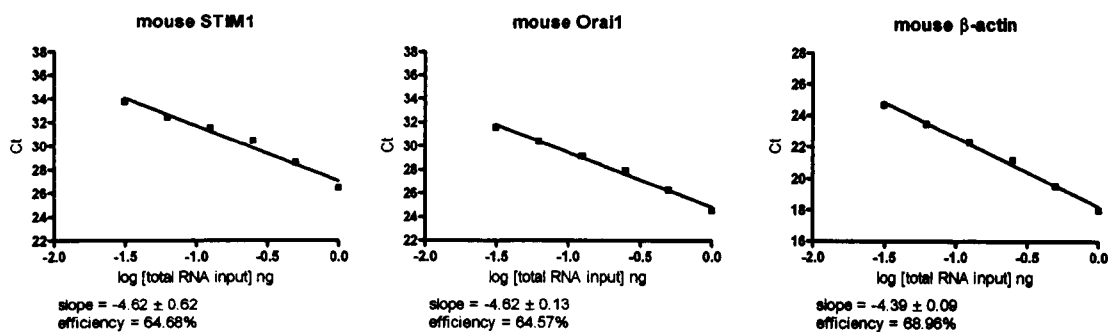
**Figure 5.3 ST2s and ST2L mRNA expression is increased in OVA sensitised lung tissue.** Quantitative PCR results showing mRNA expression of a) ST2s and b) ST2L in acute, mid-chronic and chronic OVA sensitised BALB/c mouse lung tissue compared with PBS controls. Data were analysed using the Mann Whitney test, mRNA expression is measured relative to the  $\beta$ -actin house keeping gene. Significantly increased expression of the ST2s and ST2L receptors are shown after mid-chronic and chronic OVA sensitisation of the lung tissue compared to matched non-sensitised tissue.

Results for quantitative PCR experiments comparing expression of IL-33 and the ST2 soluble (ST2s) and membrane bound (ST2L) receptors in OVA sensitised and control mouse lung tissue suggest significantly increased expression of IL-33 in all samples and increased expression of ST2s (soluble) and ST2L (full length transmembrane) in the mid-chronic and chronic OVA sensitised lung tissue compared with controls. To better understand the regulation of the IL-33/ST2 axis, further Q-PCR assays were designed for human protein targets (shown in 5.3.3).

### **5.3.2 STIM1/ Orai1: a potential role in ovalbumin sensitised airway hyper-responsiveness?**

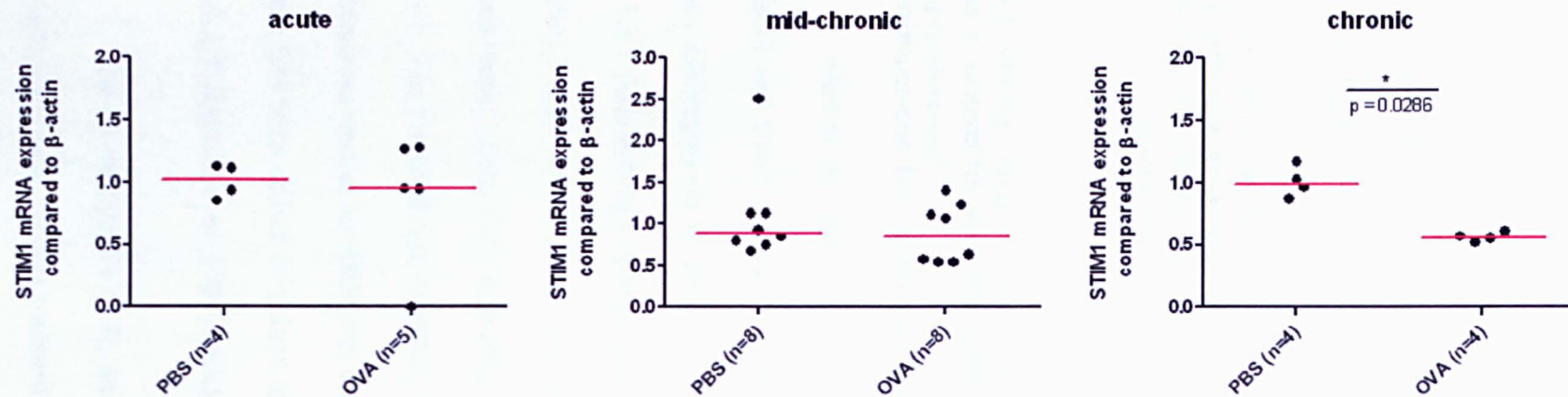
Studies on SOCC suggest a key interaction involving STIM1 (ER  $\text{Ca}^{2+}$  sensor) and Orai1 (CRAC channel pore forming subunit) (Liou, Kim et al. 2005; Roos, DiGregorio et al. 2005; Soboloff, Spassova et al. 2006). Given the data shown in 5.3.1 demonstrated upregulation of the IL-33/ST2 axis in sensitised murine lung tissue; I next investigated whether or not this resulted in altered regulation of components of the  $\text{Ca}^{2+}$  signalling pathway in the tissue, in particular STIM1 and Orai1. The potential role of SOCC and dysregulated  $\text{Ca}^{2+}$  entry in attenuating airway contraction leading to AHR was considered; given that IL-33 expressed in damaged tissue has been shown to induce other cytokines such as IL-13 (Mahn, Ojo et al. ; Chiba, Nakazawa et al. 2009) which play a role in altered  $\text{Ca}^{2+}$  handling in ASM.

Using quantitative PCR, altered mRNA expression of STIM1 and Orai1 was investigated in the OVA sensitised mouse lung tissue. Prior to Q-PCR experiments primer efficiencies were calculated for STIM1 and Orai1 assays (figure 5.4).

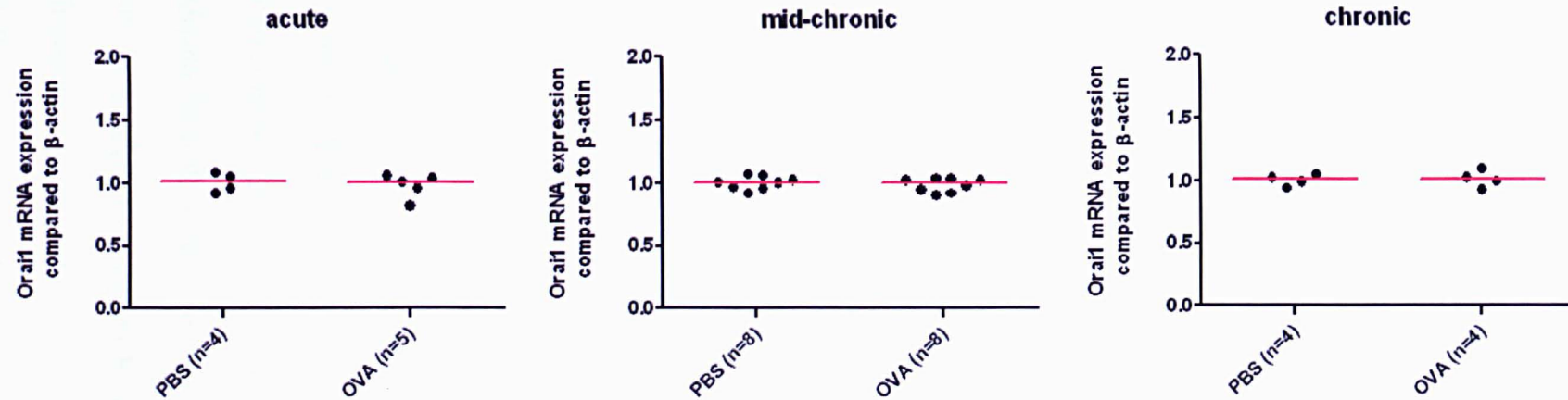


**Figure 5.4** Standard curves for Q-PCR reactions for murine *STIM1*, *Orail* and  $\beta$ -actin. For each, the amplification of two fold serial dilutions of naïve WT mouse trachea smooth muscle cDNA (three repeats within experiment) was performed. The slope of each graph and PCR amplification efficiencies are shown.

The following results for Q-PCR experiments show differences in expression of STIM1 and Orail in the OVA sensitised lung tissue and non-sensitised mouse lung tissue (figure 5.5, 5.6).



**Figure 5.5** *STIM1* mRNA expression is decreased in OVA sensitised lung tissue. Quantitative PCR results showing mRNA expression of *STIM1* in acute, mid-chronic and chronic OVA sensitised BALB/c mouse lung tissue compared with PBS controls. Data were analysed using the Mann Whitney test, with mRNA expression measured relative to the  $\beta$ -actin house keeping gene. Expression of *STIM1* was significantly decreased in chronically sensitised lung tissue ( $44 \pm 1.8\%$  decrease compared with PBS).



**Figure 5.6** No difference in *Orai1* mRNA expression between PBS and OVA sensitised lung tissue. Quantitative PCR results showing mRNA expression of *Orai1* in acute, mid-chronic and chronic OVA sensitised BALB/c mouse lung tissue compared with PBS controls. Data were analysed using the Mann Whitney test, with mRNA expression measured relative to the  $\beta$ -actin house keeping gene.

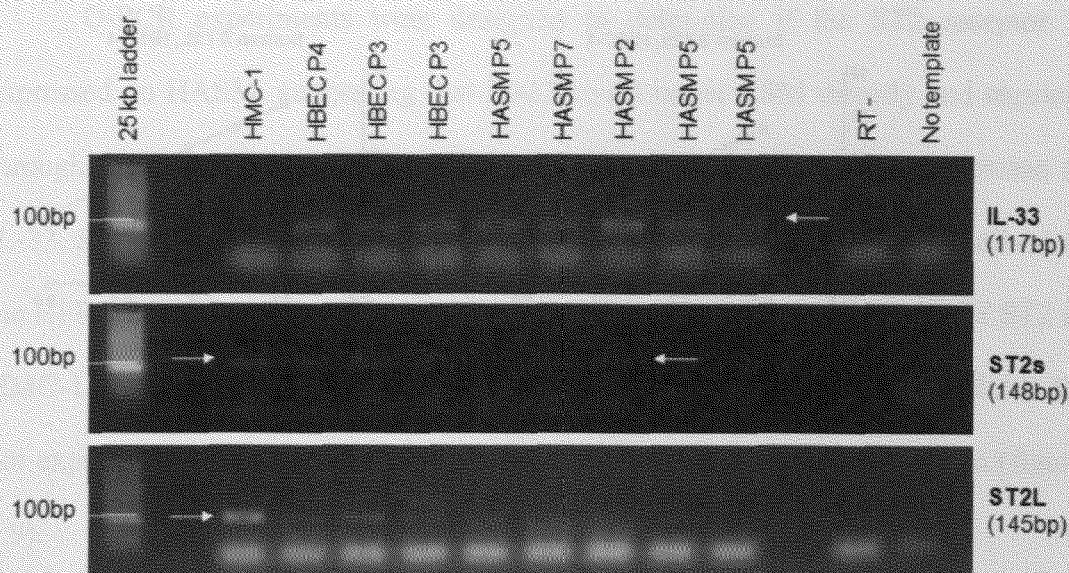


Results for quantitative PCR experiments comparing expression of STIM1 and Orai1 in OVA sensitised and non sensitised mouse lung tissue suggest STIM1 was significantly decreased in the chronic lung tissue compared with controls. Results for Orai1 expression were of particular interest as Orai1 is thought to form the CRAC channel for  $\text{Ca}^{2+}$  influx for store refilling; however, results indicated no difference in Orai1 expression between sensitised and control lung tissue, therefore this work was not pursued further in the human system. STIM1 and Orai1 have previously been demonstrated to play a role in human immunodeficiency (Feske 2009) and are known to be expressed in the ASM (Peel, Liu et al. 2008).

### **5.3.3 Expression of IL-33 and the ST2 receptor in human airway smooth muscle**

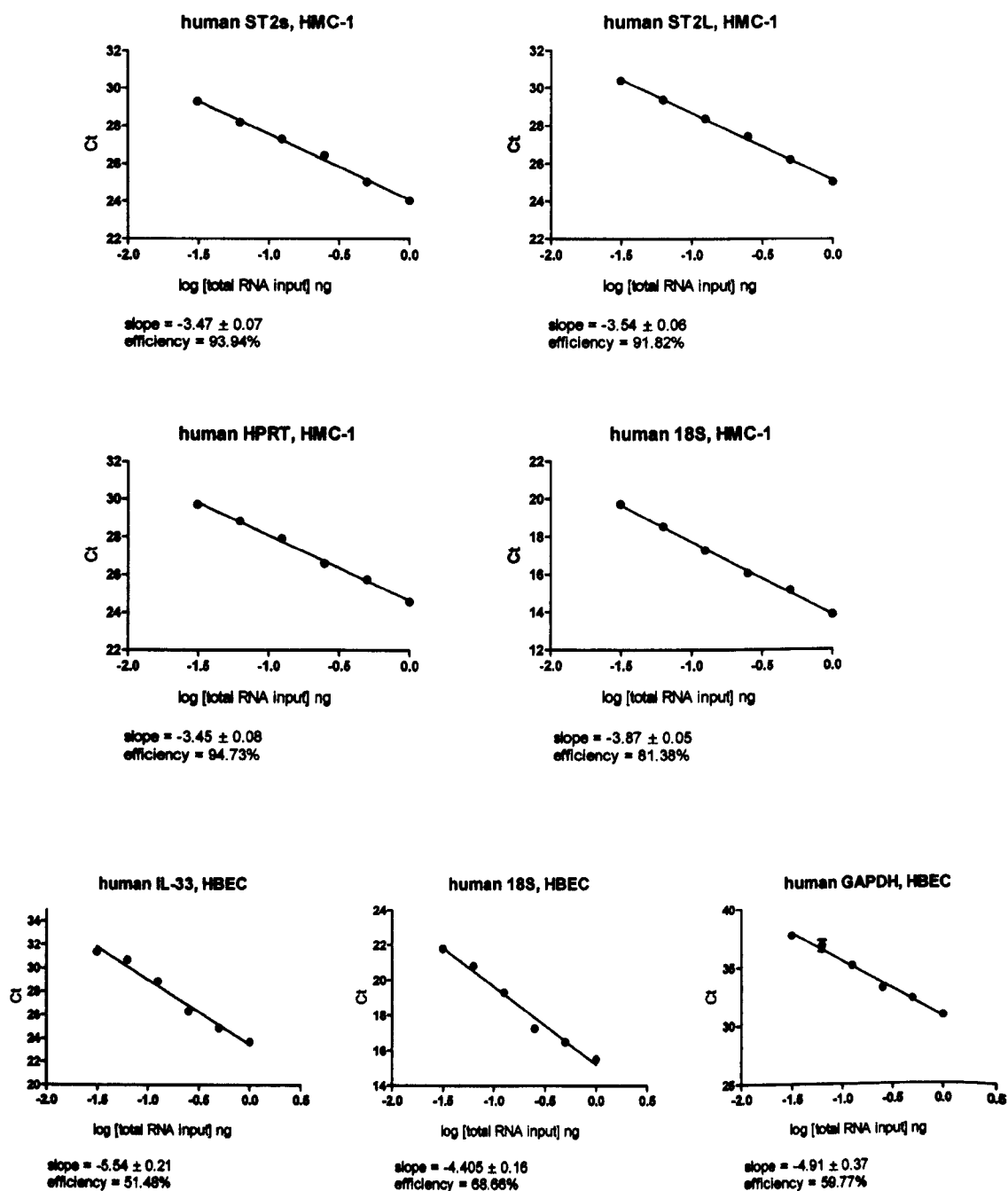
Given the previous results indicating altered expression of IL-33 and ST2 in sensitised murine lung tissue (5.3.1); Q-PCR assays were designed for human IL-33 and ST2, to determine whether IL-33 could act directly on human airway smooth muscle (HASM) to induce hyper-responsiveness. Expression of IL-33 and the ST2 membrane receptor was investigated in primary cultured HASM and human bronchial epithelial cells (HBEC) cultured from lung biopsies. The human mast cell line (HMC-1) was used as a control for ST2 expression. Initial PCR amplification of the ST2 soluble form (ST2s) (designed to have exons common to both ST2s and ST2L) and the ST2L membrane receptor, failed to produce clear or consistent bands with non quantitative visualisation using gel electrophoresis (figure 5.7).





**Figure 5.7** PCR results showing expression of IL-33, ST2s and ST2L in HMC-1, HBEC and HASM. Representative of  $n =$  seven individual repeated PCR experiments, all RT- and no template samples were free of contamination. Results suggest ST2s expression in HMC-1, HBEC and some HASM samples, however HASM expression was not consistent throughout experiment repetitions. The ST2L receptor was expressed clearly in HMC-1 and expressed at low levels in HBEC, no clear bands could be distinguished for ST2L expression in HASM. Bands for IL-33 were seen in all samples except HMC-1.  $P =$  cell passage number from initial biopsy cell isolation; bp: base pairs. Arrows indicate faint bands.

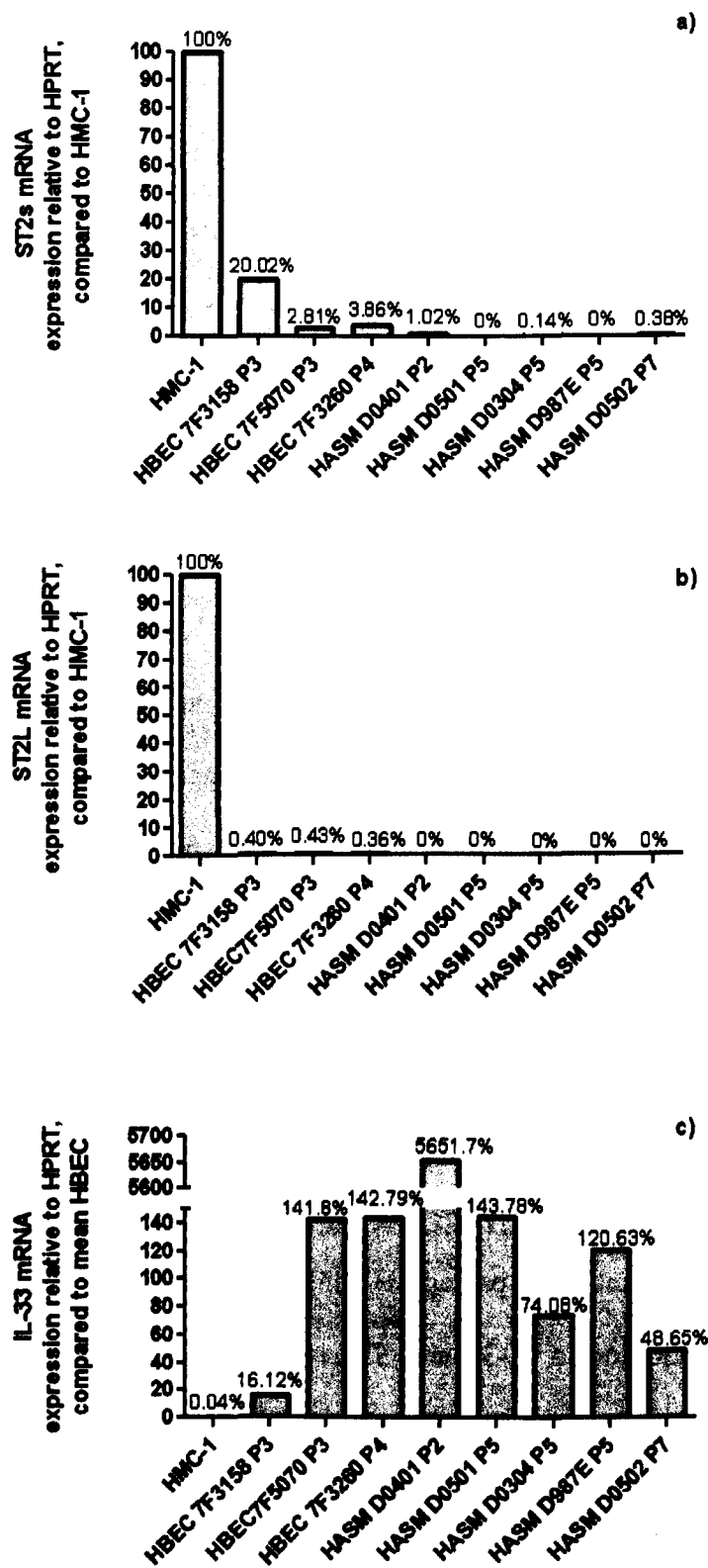
As it was difficult to clearly distinguish bands, Q-PCR was used to determine expression of ST2s and ST2L in the cell types. Q-PCR reactions were redesigned for human gene products; therefore it was also necessary to reselect a housekeeping gene. Ct values for the housekeeping genes 18S, GAPDH and HPRT were compared ( $\beta$ -actin produced higher Ct values, therefore was not considered for analysis). HPRT was deemed to have the most uniform expression between cDNA samples, therefore results shown in this section are standardised to HPRT. Primers and probes were designed using Beacon (Stratagene) software (refer to methods, 2.3). Primer efficiencies were determined by plotting a cDNA standard curve, as shown in figure 5.8. For comparative analysis the  $2^{-\Delta\Delta C_t}$  equation was used (methods, 2.3.6).



**Figure 5.8** Standard curves for Q-PCR reactions for human ST2S, ST2L, IL-33, HPRT, 18S and GAPDH for the amplification of two fold serial dilutions of either human mast cell (HMC-1) or human bronchial epithelial cell (HBEC) cDNA (three repeats within each experiment were performed). The slope of each graph and PCR amplification efficiencies are shown.

Q-PCR experiments were designed to determine if the ST2 receptor is expressed on HASM, suggesting that IL-33 could target ASM directly and regulate through this pathway. For all Q-PCR results shown in figure 5.9 for expression of IL-33, ST2s and ST2L in HMC-1, HBEC and HASM, Ct values were normalised to the HPRT house keeping gene. All ST2 data was normalised to expression of ST2 in HMC-1, given the consistent expression in these samples. However, as HMC-1 did not express IL-33, all data for IL-33 is compared to mean HBEC expression (figure 5.9).

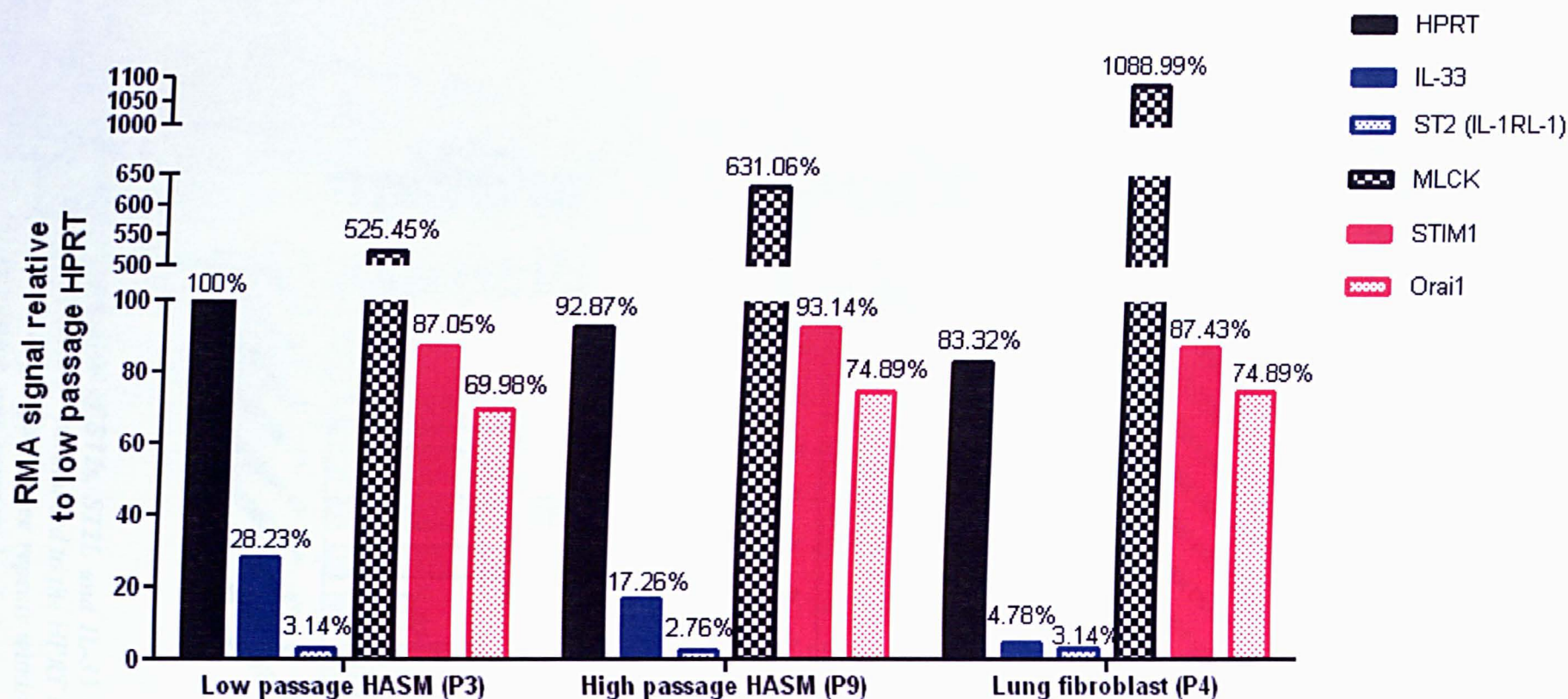
These Q-PCR results shown in figure 5.9 suggested potential cell passage related decreases in expression of IL-33 and the ST2 receptors. To support these results, previous data from within our department was searched for IL-33 and the human ST2 receptor (IL-1RL-1) in an Affymetrix Genechip Human Gene 1.0 ST array data set designed for 28,869 well annotated genes with 764,885 distinct probes (Singh, Billington et al. 2010). Raw data was extracted from the scanned images to generate normalised probe-level intensity measurements for the Affymetrix gene chips termed robust multi-array average (RMA). Raw RMA values for the target genes were compared relative to the HPRT housekeeping gene to reflect expression levels in low and high passage HASM and lung fibroblasts (passage four) (figure 5.10). In addition to IL-33 and ST2 receptor (IL-1RL-1), STIM1 and Orai1 were included in the search, as was myosin light chain kinase (MLCK) which served for comparison as it is known to be expressed at high levels in ASM.



**Figure 5.9** Quantitative expression of ST2s, ST2L and IL-33 in HMC-1, HBEC and HASM. All Q-PCR Ct values were normalised to the HPRT house keeping gene (n = two experiments for each target gene, three repeats within each experiment). ST2s (a) and ST2L (b) expression was compared relative to HMC-1 (shown as

100%). ST2s and ST2L were clearly expressed in HMC-1 and expressed at low levels in HBEC. There was very low expression of ST2s in one HASM donor. Expression of ST2L in HASM was undetectable. c) As IL-33 was expressed at very low levels in HMC-1, mean values for HBEC expression served as a standard (100% expression) to compare samples. IL-33 expression was detected in HBEC and in HASM, often at very high levels. It is noted that the low passage (HASM D0401 P2) sample showed expression of ST2s (Ct 37) and significantly high expression of IL-33; suggesting donor/passage number variability. P = cell passage number.





**Figure 5.10** Probe level intensity measurements generated from Affymetrix data chips searched for expression of HPRT, IL-33 and the ST2 receptor (IL-1RL-1), MLCK, STIM1 and Orai1 in low and high passage HASM and lung fibroblasts. Results are shown relative to the HPRT housekeeping gene in low passage HASM. Results of interest indicate very low expression of the ST2 receptor in low and high passage HASM and decreased expression of IL-33 in higher passage HASM. HPRT: Hypoxanthine phosphoribosyltransferase (housekeeper); MLCK: Myosin light chain kinase; IL-1RL-1: IL-1 receptor like 1; STIM1: Stromal Interaction molecule 1. P = cell passage number.



## 5.4 DISCUSSION

Given the results in the previous chapter suggesting that the IL-33/ST2 interaction plays a role in the development of AHR in a mouse model of allergic airway disease; in this chapter Q-PCR experiments were designed to investigate alterations in expression level of IL-33 and the ST2 soluble (ST2s) and membrane (ST2L) forms of receptor in acute, mid-chronic and chronic OVA sensitised murine lung tissue. As previous studies have found IL-13 mediated dysregulation of the  $\text{Ca}^{2+}$  sensitive pathway in ASM leads to AHR (Chiba, Nakazawa et al. 2009); additional Q-PCR experiments were designed to investigate alterations in mRNA expression of STIM1 and Orai1 (key store operated calcium channel components involved in  $\text{Ca}^{2+}$  influx in ASM). Q-PCR results revealed increased mRNA expression of IL-33, the ST2 soluble and ST2 membrane receptor in acute, mid-chronic and chronic OVA sensitised BALB/c mouse lung tissue. There was no significant difference in Orai1 mRNA expression between control and OVA sensitised mice. However, after chronic sensitisation STIM1 expression was significantly decreased; as experiments were not followed further, no explanation was reached for these results.

Q-PCR experiments were also performed to look for expression of IL-33 and the soluble and membrane forms of the ST2 receptor (also named IL-1RL-1 in human) in human airway smooth muscle (HASM), human airway epithelial cells (HBEC) and a human mast cell line (HMC-1). Results indicate that IL-33 is expressed at very low levels in HMC-1 and clearly expressed in HASM (passage two - seven) and HBEC (passage three - four). The ST2 soluble receptor (ST2s) was highly expressed in HMC-1, expressed at very low levels in HBEC and was virtually undetectable in HASM. The ST2L membrane receptor was expressed at high levels

in HMC-1, detectable at low levels in HBEC, but absent in HASM. These targets were also searched for in our departmental data set for Affymetrix Gene chip analysis of ~ 30,000 genes for expression in low and high passage HASM and lung fibroblasts (Singh, Billington et al. 2010). Results confirmed the very low expression of the ST2 receptor in low and high passage HASM and decreased expression of IL-33 in higher passage HASM.

#### **5.4.1 Altered IL-33 and ST2 mRNA expression in ovalbumin sensitised mouse lung tissue**

Quantitative PCR results showed significantly increased expression of IL-33, the ST2s and ST2L membrane bound receptor in the OVA sensitised lung tissue. These results are consistent with recent papers finding increased IL-33 expression in bronchial biopsies of asthmatic patients (Prefontaine, Nadigel et al. 2010; Prefontaine, Lajoie-Kadoch et al. 2009) and increased serum ST2 levels that correlate with asthma severity (Oshikawa, Kuroiwa et al. 2001). The IL-33/ST2 receptor's role in development, maintenance of the allergic response and AHR is also supported in a study by Kearley and colleagues, 2009, using OVA sensitised BALB/c and C57BL/6 mice and ST2, IL-4 and IL-13 blocking Ab to confirm the role of T<sub>H</sub>2 cells, particularly IL-33 in maintaining AHR (Kearley, Buckland et al. 2009).

Quantitative PCR to investigate altered mRNA expression of STIM1 and Orail in the samples revealed interesting results. My initial hypothesis was that increased expression of these proteins might result in increased Ca<sup>2+</sup> pools for

maintenance of contraction to support AHR. However, the results obtained suggested no difference in Orai1 expression between control and OVA sensitised mice from the acute, mid-chronic and chronic models. There was no difference in STIM1 expression between control and sensitised mice after acute and mid-chronic sensitisation, however, STIM1 mRNA expression was significantly decreased after chronic sensitisation. Whilst this might reflect the relatively small number of samples available for study, it is also possible that STIM1 expression is reduced following long term sensitisation on a negative feedback homeostasis mechanism. This requires further study.

In this thesis, the role of SOCC has been investigated within the context of ASM homeostasis and contraction pathways. However, there is evidence for STIM1 and Orai1 playing a role in immunodeficiency, inflammation and infection (Feske 2009) through T cell activation. SOCC activated prolonged  $\text{Ca}^{2+}$  entry through CRAC channels activates  $\text{Ca}^{2+}$  sensitive transcription factor NFAT, responsible for directing T cell proliferation and gene expression (Macian 2005; Di Sabatino, Rovedatti et al. 2009). Altered  $\text{Ca}^{2+}$  responses in T cells have been associated with such autoimmune and inflammatory conditions as systemic lupus erythematosus (Tenbrock, Juang et al. 2007), rheumatoid arthritis (Feske 2007), inflammatory bowel disease (Di Sabatino, Rovedatti et al. 2009) and most notably severe combined immunodeficiency syndrome in patients lacking CRAC (Feske, Prakriya et al. 2005). Perhaps the inhibition of SOCC in the T cell inflammatory response may present another direction for therapeutic intervention in airway inflammation.

### 5.4.2 IL-33 sources and targets in human airway inflammation

Having demonstrated the importance of the IL-33/ST2 axis in murine lung inflammation following OVA sensitisation, I proceeded to investigate the axis in primary human airway cells relevant to human lung disease. Quantitative PCR experiments revealed that HBEC and HASM were a source of IL-33, however the ST2L membrane receptor was undetectable on HASM (five patient donors) and detected at very low levels on HBEC (three patient donors). Given that these studies were conducted in cultured cells from passage two - seven, the potential for receptor expression to disappear in culture was considered (ST2s expression was noted in the HASM passage two sample, but not seen in higher passage cells). Without access to fresh HASM, ST2L membrane receptor expression on ASM could not be studied further. No other studies have confirmed expression of ST2L protein on ASM (summarised in table 5.2). These results were further supported by results generated from the Affymetrix gene array analysis data; the ST2 receptor was barely detectable in low and high passage HASM and lung fibroblasts. IL-33 was detectable in low passage HASM and expression was decreased in high passage HASM, IL-33 was also expressed at very low levels in lung fibroblasts.

During these experiments it became increasingly apparent that such structural cells as airway smooth muscle and endothelial cells may serve as a source of IL-33 and in turn target the ST2L receptor expressed on inflammatory cells leading to further production of cytokines known to act directly on the ASM. In human, IL-33 is predominantly expressed by epithelial cells, endothelial cells (Moussion, Ortega et al. 2008) and fibroblasts (Sanada, Hakuno et al. 2007) at the protein level. IL-33 targets cells expressing the ST2 membrane receptor, notably  $T_H2$  cells rather than

T<sub>H</sub>1 (Xu, Chan et al. 1998). IL-33 is shown to induce such proinflammatory cytokines and chemokines as IL-5, IL-13, IL-1, IL-6, TNF- $\alpha$ , CCL2 and CCL3 (Ali, Huber et al. 2007; Allakhverdi, Smith et al. 2007; Iikura, Suto et al. 2007; Moulin, Donze et al. 2007; Kurowska-Stolarska, Kewin et al. 2008). In addition IL-33 induces degranulation of mast cells and enhances mast cell maturation and survival.

Quantitative PCR studies in this chapter suggested an absence of the ST2L membrane receptor on ASM from cultured HASM; no previous literature suggesting ST2L expression in ASM cells was found. This suggests that the effects of IL-33 may be indirect and secondary cytokines may be produced after cell stimulation with IL-33; for potential cytokine candidates shown to alter ASM Ca<sup>2+</sup> homeostasis and induce AHR see table 5.3. In work using a neutralising Ab targeted against the human ST2 receptor which would be effective both against ST2 on the ASM cell surface or in soluble form in the culture media, IL-33 ELISA on the supernatants collected from cells suggested the ST2 receptor did not play a significant role in IL-33 release on ASM cells (Prefontaine, Lajoie-Kadoch et al. 2009). These data could also support the notion that IL-33 may play an intracellular role in altering gene transcription, like other IL-1 type cytokines (Gadina and Jefferies 2007).

**Table 5.3 Cytokines implicated in the induction of altered airway smooth muscle  $Ca^{2+}$  homeostasis and induced airway hyper-responsiveness.**

Cytokine	Potential role in airway hyper-responsiveness	Reference
IL-13	In ASM IL-13 modulates the CD38/cADPR-dependent pathway influencing $Ca^{2+}$ signalling. SERCA expression was decreased after cytokine exposure and rate of fall of $[Ca^{2+}]_i$ transients was slowed. IL-13 produced as a T lymphocyte product was shown to act directly on ASM to induce hyper-responsiveness. IL-13 was also shown to induce upregulation of the RhoA protein in human bronchial ASM and contributed to AHR through the RhoA pathway in mice.	(Venkayya, Lam et al. 2002; Deshpande, Dogan et al. 2004; Chiba, Nakazawa et al. 2009; Sathish, Thompson et al. 2009)
IL-8	IL-8 expression is increased in patients with cystic fibrosis and asthma, and is a CXC chemokine and chemo attractant for neutrophils by CXCR1 and CXCR2 G protein coupled receptors. Constitutive expression of CXCR1 and CXCR2 was shown in HASM, IL-8 mediated $Ca^{2+}$ release, contraction and migration.	(Govindaraju, Michoud et al. 2006)
TGF- $\beta$	TGF- $\beta$ increases ASM contraction to bradykinin in mouse tracheal rings.	(Kim, Jain et al. 2005)
TNF- $\alpha$	TNF- $\alpha$ stimulates expression of intercellular adhesion molecule 1 (ICAM1) by activating NF- $\kappa$ B. TNF- $\alpha$ induced expression of ICAM1 on ASM cells involves a thapsigargin sensitive pathway. SERCA expression was decreased after cytokine exposure and rate of fall of $[Ca^{2+}]_i$ transients was slowed. Results suggest that inflammation maintains $[Ca^{2+}]_i$ levels by decreasing SERCA expression and slowing $Ca^{2+}$ reuptake (SERCA regulation involves CaMKII).	(Amrani, Lazaar et al. 2000; Sathish, Thompson et al. 2009)
IL-1 $\beta$	Reported to desensitise histamine $H_1R$ , involving activation of p38 MAPK and NF- $\kappa$ B, inducing expression of COX-2 and PGE <sub>2</sub> synthesis. PGE <sub>2</sub> increased intracellular cAMP leading to PKA activation which phosphorylated and subsequently functionally uncoupled histamine $H_1R$ . Results from this study suggest that bronchial hyper-responsiveness to histamine is not associated with cytokine-induced enhancement in $H_1R$ signaling.	(Pype, Xu et al. 2001)
IL-4	IL-4 produced as a T lymphocyte product was shown to act directly on mouse ASM to induce hyper-responsiveness.	(Venkayya, Lam et al. 2002)

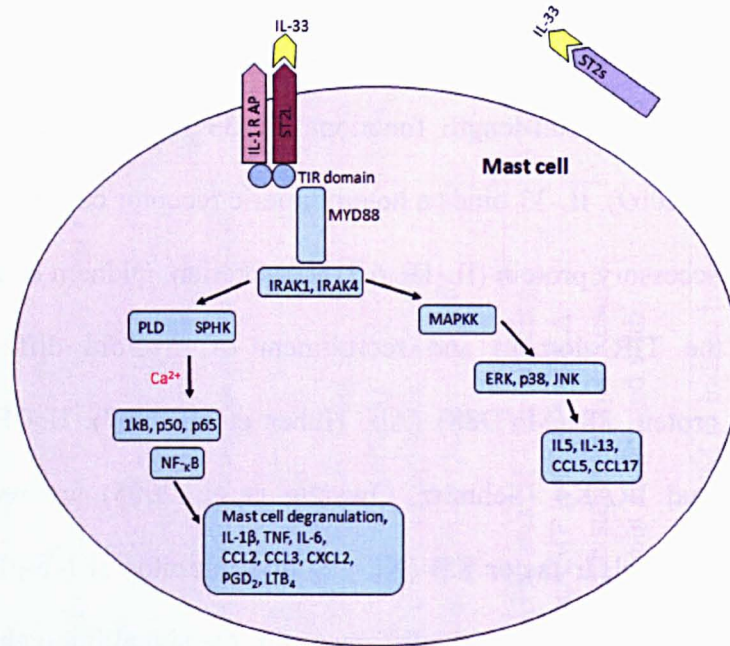


### 5.4.3 IL-33: mechanisms of action

In the absence of proinflammatory stimuli IL-33 localises to the nucleus (Baekkevold, Roussigne et al. 2003; Carriere, Roussel et al. 2007). Cellular necrosis leads to release of full-length functional IL-33 (Cayrol and Girard 2009; Luthi, Cullen et al. 2009). IL-33 binds a heterodimeric receptor complex involving ST2 and an IL-1R accessory protein (IL-1R AP) (Chackerian, Oldham et al. 2007). Signalling requires the TIR domain and recruitment of myeloid differentiation primary-response protein 88 (MYD88) (Ali, Huber et al. 2007). IL-1R associated kinase (IRAK1) and IRAK4 (Schmitz, Owyang et al. 2005) are recruited and induce activation of nuclear factor KB (NF- $\kappa$ B) and inhibitor of NF- $\kappa$ B- $\alpha$  (I $\kappa$ B $\alpha$ ). Various studies have noted cell specific differences in the signalling pathways. The pathway activated by IL-33 in mast cells are shown in figure 5.11.

In mast cells, IL-33 activates phospholipase D and sphingosine kinase and through Ca<sup>2+</sup> mobilisation, targets NF- $\kappa$ B. This pathway is required for the production of IL-1 $\beta$ , IL-3, IL-6, TNF- $\alpha$ , CXC chemokine ligand 2 (CXCL2), CCL2, CCL3, prostaglandin D<sub>2</sub> and leukotriene B<sub>4</sub> (Allakhverdi, Smith et al. 2007; Moulin, Donze et al. 2007; Pushparaj, Tay et al. 2009). An alternative pathway involves mitogen activated protein kinase (MAPK), through extracellular signal-related kinase 1 (ERK1), p38 and JUNN-terminal kinase-1 (JNK-1) (Pushparaj, Tay et al. 2009), leading to production of IL-5, IL-13, CCL5, CCL17 and CCL24 (Kurowska-Stolarska, Kewin et al. 2008). Studies involving IgE priming show increased cell surface expression of ST2L and induction of mast cell degranulation (Pushparaj, Tay et al. 2009). Interestingly, the soluble isoform ST2s receptor acts as a decoy receptor

to bind excess IL-33 and inhibit binding to the ST2L membrane receptor (Hayakawa, Hayakawa et al. 2007).



**Figure 5.11 IL-33 signalling in mast cells.** IL-33 signals through an ST2L receptor and IL-1 receptor accessory protein (IL-1RAP) complex, recruiting MYD88. Through a pathway involving phospholipase D (PLD)/ sphingosine kinase (SPHK) activated  $\text{Ca}^{2+}$  mobilisation, nuclear factor K B ( $\text{NF-}\kappa\text{B}$ ) is activated leading to further mast cell degranulation, cytokine and chemokine production. An alternative pathway involving mitogen activated protein kinase (MAPK) also leads to production of IL-13, IL-5 and other chemokines. MYD88: Myeloid differentiation primary-response protein 88; IRAK1, IRAK4: IL-1R associated kinase;  $\text{I}\kappa\text{B}\alpha$ : inhibitor of  $\text{NF-}\kappa\text{B-}\alpha$ ; ERK1: extracellular signal-related kinase 1; JNK-1: JUNN-terminal kinase-1; CCL, CC, CXCL, CXC: chemokine ligand; TNF: tumour necrosis factor;  $\text{PGD}_2$ : prostaglandin  $\text{D}_2$ ;  $\text{LTB}_4$ : leukotrine  $\text{B}_4$ . Adapted from (Liew, Pitman et al.).

IL-33 targets a range of immune cells and may play a role in a wide variety of disease states such as arthritis (Xu, Jiang et al. 2008), cardiovascular disease (Weinberg, Shimp et al. 2002), allergy and anaphylaxis (Pushparaj, Tay et al. 2009), Alzheimer's disease (Chapuis, Hot et al. 2009) and respiratory syncytial virus infection (Walzl, Matthews et al. 2001). Given that IL-33 is released from structural

cells like the respiratory epithelium, it may serve as an initial defence against pathogens or may play a role in sensing damage by infection or inflammation.

Studies in Chapter Four indicated that treatment of naïve mouse lung slices with IL-33 (100ng/ml, over night exposure) lead to AHR to MCh despite a non inflammatory environment present; through a pathway that required interaction with the ST2R. Experiments in this chapter were designed to determine whether IL-33 acts directly on the ASM via targeting of the ST2R. Results indicate that HASM does not express the ST2R. Therefore, IL-33 may act on other non inflammatory cell types present that express the ST2R which inturn produce cytokines that target the ASM directly to alter airway responsiveness. Alternatively, IL-33 is shown to be expressed on ASM and may act internally by translocating to the nucleus and altering transcription of other cytokines that influence the contractile pathway.

Future experiments to investigate key cytokine candidates involved in AHR include quantitative PCR experiments performed on OVA sensitised and cytokine treated mouse lung tissue to investigate alterations in mRNA expression of key signaling pathway members involved in the contractile pathway in ASM. Further investigation is required to identify the unknown cell types present expressing the ST2R which IL-33 may act on to produce further cytokine production for down stream AHR of ASM (refer to 6.2).

In summary, this chapter aimed to explore some of the mechanisms involved in IL-33 mediated AHR. The results obtained showed increased mRNA expression of IL-33, the ST2 soluble and membrane receptor in OVA sensitised BALB/c mouse

lung tissue; indicating a role for IL-33/ST2 receptor in tissue damage and airway inflammation. Investigation of IL-33, the ST2 soluble and membrane bound receptor expression in human airway smooth muscle (HASM), human bronchial epithelial cells (HBEC) and mast cells revealed that epithelial and ASM are a source of IL-33. However, ST2 receptor expression was virtually undetectable in these cell types, yet highly expressed in mast cells. Together these results support previous literature suggesting the possible role for IL-33 as a sensor of tissue damage and driver of the  $T_H2$  inflammatory response through inflammatory cell activation. While the role, release and downstream pathways activated by IL-33 in asthma and various inflammatory states require further studies; these data indicate the potential for therapeutic intervention by targeting of IL-33 and the ST2 receptors.

## **CHAPTER SIX**

### **DISCUSSION**



## **6. CHAPTER SIX**

The aims of the work described in this thesis were:

- To optimise the use of the precision cut lung slice model for the study of airway contraction and relaxation.
- To examine key pathways involved in contractile signalling via  $\text{Ca}^{2+}$  influx.
- To investigate altered contractile responses in a murine model of OVA-induced allergic airway sensitisation using precision cut lung slices.
- To define key mediators involved in sensitisation of airway smooth muscle in sensitised airways.
- To develop the use of porcine precision cut lung slices as a bridging model to help develop human studies.

### **6.1 Summary of results**

In this thesis my experiments were initially designed to establish the lung slice technique in mouse and in pig to provide a bridging model for future human studies. Selected pharmacological agents were used to target specific channels involved in the  $\text{Ca}^{2+}$  signalling pathway required for contraction induction and maintenance. The results obtained demonstrated the significance of extracellular  $\text{Ca}^{2+}$  for internal  $\text{Ca}^{2+}$  store refilling and release to repeatedly induce and maintain a contraction and showed interesting species differences in agonist and inhibitor sensitivity; with the porcine system sharing similar pharmacology to that reported in human airways. These results highlight the necessity for regulated  $\text{Ca}^{2+}$  handling in ASM for correct



tone maintenance. Although the results in this thesis suggest that under normal physiological conditions, short term, SOCC do not contribute to regulation of intracellular  $\text{Ca}^{2+}$  stores; an alternative approach in the future would be to explore the importance of SOCC long term for maintenance of intracellular  $\text{Ca}^{2+}$  stores. The species differences shown in ASM  $\text{Ca}^{2+}$  handling and pharmacological response suggest that conservative assumptions need to be made for human airway behaviour based on findings in animal models and demonstrate the need for human studies at the peripheral airway level.

The lung slice technique was next applied to a sensitised mouse model of airway inflammation; experiments were designed to investigate the key inflammatory mediators involved in AHR and the  $\text{T}_\text{H}2$  inflammatory process. Results revealed that OVA sensitisation led to progressive structural remodelling and AHR to MCh. However, this hyper-responsiveness was decreased 48 hours post lung removal despite persistent airway remodelling. In WT naïve mice, IL-33 exposure significantly increased AHR to MCh. However, in lung slices prepared from ST2 KO mice IL-33 was unable to sensitise the contractile response *in vitro*. These results illustrate a key role for IL-33 in the induction of hyper-responsiveness in this murine model and demonstrate the contribution of the inflammatory environment to persisting increased airway sensitivity, independent of structural remodelling. Experiments designed to further investigate the mechanisms involved in IL-33 mediated AHR revealed increased mRNA expression of IL-33 and also the ST2 soluble and membrane receptor in the OVA sensitised mouse lung tissue. Investigation in a cultured human airway system for IL-33, ST2 soluble and membrane bound receptor expression showed that epithelial and ASM cells are a source of IL-33. However, ST2 receptor expression was undetectable in these cell

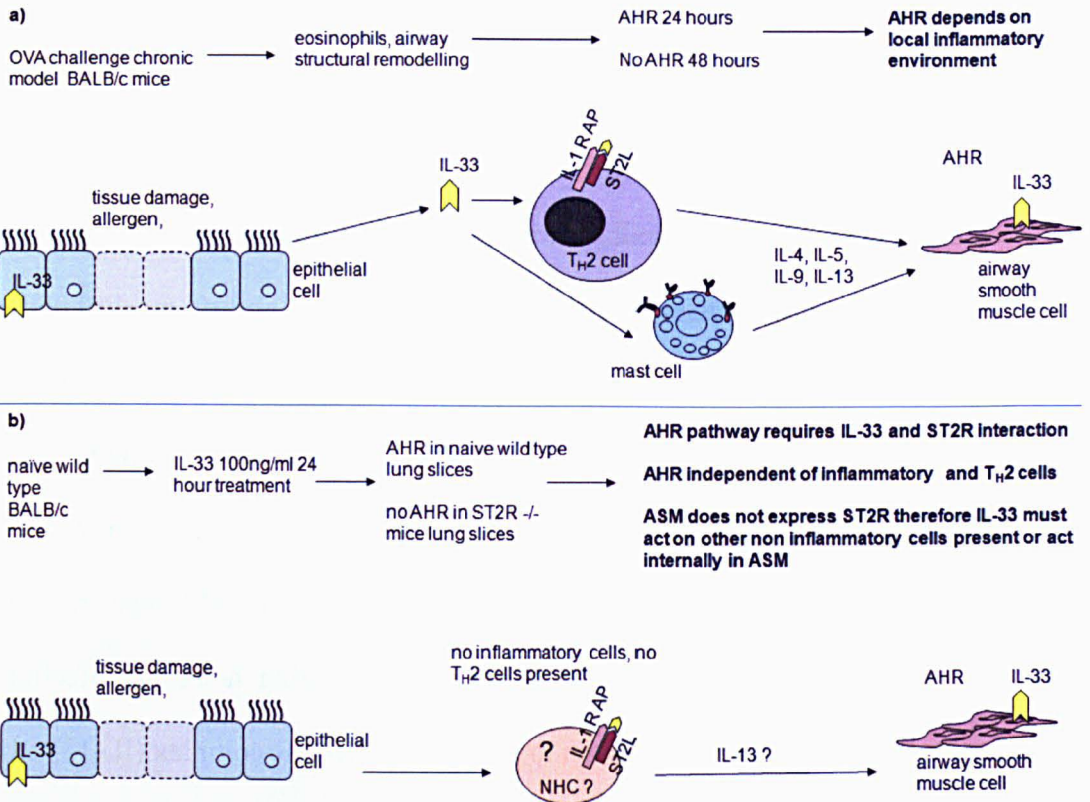
types, yet highly expressed in mast cells. These results support the notion that IL-33 may function as an initiator of the inflammatory response in tissue injury (discussed further in 6.2) and also highlight the need to explore the effects of endogenous IL-33 administration on human lung slices and the potential therapeutic role of the soluble form of the ST2 receptor in preventing T<sub>H</sub>2 cytokine induction.

## 6.2 IL-33 initiates the pathway for AHR

Experiments in Chapter Four demonstrated that overnight treatment with IL-33 (100ng/ml) induced AHR in naïve WT type mouse lung slices, yet this increased response was abolished in ST2R <sup>-/-</sup> mice indicating that the ST2R is required for the signaling pathway leading to AHR. Results in Chapter Five indicated that human ASM does not express the ST2R suggesting that the pathway to AHR is not via direct activation of the ASM but may involve activation of inflammatory cells present or other helper cells that lead to production of cytokines that in turn act directly on the ASM to alter the signaling pathway leading to AHR (figure 6.1). Alternatively, these results may suggest that IL-33, which is shown to be expressed on ASM, may act locally to alter transcription, promoting production of other cytokines which influence the contractile response.

Studies using the recently generated IL-33 <sup>-/-</sup> mouse model indicate that IL-33 plays a role in innate inflammation in the lung and gastrointestinal tract. However, is not necessary for further acquired immunity (Oboki, 2011). Further support of the key role of IL-33 in the initial induction of the innate response, was shown in a mouse model of influenza infection induced AHR that was independent of T<sub>H</sub>2 cells and adaptive immunity, through a pathway that required IL-33 acting on

cells of non T, non B cell origin, namely natural helper cells (NHC) (Chang, Kim et al. 2011). Further more the study demonstrated that natural helper cells were present prior to infection. Natural helper cells express IL-7R (TSLPR), ST2R, IL-17RB (IL-25R), express C-kit (mast cell growth factor), have high levels of MHC class II, can function as antigen presenting cells and have the ability to differentiate into macrophage, mast cells, eosinophils and basophils (Chang, Kim et al. 2011; Cormier and Kolls 2011; Kim, DeKruyff et al. 2010; Moro, Yamada et al. 2009; Neill, Wong et al. 2010; Saenz, Siracusa et al. 2010). Studies suggest that IL-33 may act on NHC to produce such cytokines as IL-13 which plays a role in ASM hyper-reactivity. Both IL-33 and IL-25 act on NHC to produce IL-13 during helminth infection (Price, Liang et al. 2010). ASM is shown to express the IL-13R complex (IL-13R $\alpha$ 1 and IL-4R $\alpha$ ) (Moynihan, Tolloczko et al. 2008). IL-13 has also been shown to alter the calcium sensitivity pathway in ASM (Chiba, Nakazawa et al. 2009). Furthermore, in IL-13 $^{-/-}$  mice that were sensitised and challenged, despite airway inflammation and eosinophilia present, AHR does not develop (Walter, McIntire et al. 2001). Collectively these studies may implicate IL-33 as a critical initiator of the innate response, with the ability to act on non inflammatory natural helper cells to inturn produce key cytokines such as IL-13 which acts on the ASM to influence airway contractility (figure 6.1).



**Figure 6.1 Possible IL-33 induced pathways in airway hyper-responsiveness.** *a)* Allergen exposure (via OVA sensitisation) or tissue injury to such structural barriers as epithelial cells leads to release of IL-33. IL-33 drives the T<sub>H</sub>2 inflammatory response by targeting the ST2L receptor on T<sub>H</sub>2 and mast cells to up regulate transcription of cytokines and chemokines that contribute to airway remodelling and airway hyper-responsiveness. However, in these studies *b)* AHR was also shown in naïve mouse lung slices, independent of inflammatory cells or T<sub>H</sub>2 cells suggesting that IL-33 (100ng/ml, 24 hour treatment) must act on other cell types present in the local environment to induce AHR. As ASM does not express the ST2R, IL-33 may act on a non inflammatory cell present such as a natural helper cell (NHC) or act directly on ASM to alter transcription of other cytokines such as IL-13. IL-1RAP: IL-1 receptor accessory protein.

### 6.3 IL-33: a danger signal?

Recent theories surrounding the activation of the immune response by a ‘danger signal’ (also termed ‘alarmin’) are supported by the discovery of receptors for pathogen associated molecular patterns (PAMP), such as toll-like receptors (TLR), nucleotide-binding oligomerization domain (NOD)-like receptors and C-type lectin receptors and further supported by genetic mutations in these pathways being

related to chronic non infectious inflammatory diseases (Brydges and Kastner 2006; Fritz, Ferrero et al. 2006; Petrilli, Dostert et al. 2007). Germ line encoded pattern recognition receptors and recognition of highly conserved PAMP provides an intrinsic system to identify pathogen types and generate the required immune response (Akira, Uematsu et al. 2006). IL-33 has been suggested as an 'alarmin' (Moussion, Ortega et al. 2008) due to its ability to recruit and activate immune cells, nuclear expression profile and its secretion by necrotic cells; all similarities between IL-33 and such other 'alarmins' as high mobility group box 1 (HMGB1) and IL-1 $\alpha$  (Oppenheim and Yang 2005; Bianchi 2007) (discussed below).

IL-33 expression was initially observed in endothelial cells of high endothelial venules (HEV), specialised blood vessels for the recruitment of lymphocytes to lymphoid organs (Baekkevold, Roussigne et al. 2003; Carriere, Roussel et al. 2007). IL-33 is now the most recent addition to the IL-1 family of cytokines; known to play a major role in inflammatory, infectious and autoimmune disease (Dinarello 1996). Predominantly clustered on human chromosome two (Sims, Nicklin et al. 2001), family members include IL-1 $\alpha$ , IL-1 $\beta$ , IL-1Ra (IL-1 receptor antagonist) and IL-18. The expression of these cytokines is tightly regulated by alternatively spliced forms of ligands and receptors, soluble receptors (IL-1 receptor type 2) and natural antagonist proteins (IL-Ra, IL-18 binding protein) (Dunne and O'Neill 2003). IL-1 cytokines act through the toll-like receptor-IL-1 receptor super family, characterised by the presence of an intracellular toll-IL-1R (TIR) module. IL-1 ligands typically bind to a receptor complex consisting of two IL-1 receptor family members (e.g the receptor complex for IL-1 $\alpha$  and IL-1 $\beta$  involves IL-1R1 and IL-1RAP (receptor accessory protein, the same receptor required for the IL-33/ST2 membrane bound complex)) (Dunne, Ejdeback et al.

2003). IL-1 receptor signalling pathways typically involve activation of NF- $\kappa$ B and the mitogen activated protein kinases (MAP) p38, JNK and ERK, as described for IL-33 (Schmitz, Owyang et al. 2005).

IL-33 is suggested to play a role in response to tissue injury and infection based on its expression in the nucleus of endothelial and epithelial cells from tissues where pathogens and allergens may be encountered. Nuclear localisation occurs via an amino-terminal homeodomain-like DNA binding motif (Baekkevold, Roussigne et al. 2003) where IL-33 shows transcriptional regulator properties and associates with chromatin; initially demonstrated in the nucleus of HEV *in vivo* (Carriere, Roussel et al. 2007; Roussel, Erard et al. 2008). Studies suggest dual roles for IL-33 as a cytokine and intracellular nuclear factor similar to the cytokines IL-1 $\alpha$  and chromatin associated cytokine HMGB1. IL-1 $\alpha$  exerts proinflammatory cytokine activation through IL-1R1 and IL-1RAP (Dinarello 1996) and also translocates to the nucleus to regulate transcription (Werman, Werman-Venkert et al. 2004). HMGB1 acts as a proinflammatory cytokine secreted by macrophages during inflammation (Bonaldi, Talamo et al. 2003), after tissue injury and cell necrosis (Scaffidi, Misteli et al. 2002; Lotze and Tracey 2005) and also regulates transcription, chromatin structure and function in the nucleus (Agresti, Lupo et al. 2003).

Although the role of IL-33 activation of the ST2 receptor on T<sub>H</sub>2 and mast cells is well documented, the nuclear function of proIL-33 and how IL-33 is released from the nucleus for secretion remains unclear. IL-33 is produced as a 31KDa propeptide, similar to IL-1 $\beta$  and IL-18 and is cleaved to generate the mature 18KDa form (Carriere, Roussel et al. 2007; Moulin, Donze et al. 2007). *In vitro* studies have reported cleavage of proIL-33 through mechanisms involving either the nucleotide



binding domain and leucine rich repeat containing gene family (NLR) P3 or NLRC4 inflammasomes, possibly involving caspase-1 (Schmitz, Owyang et al. 2005). However, the caspase-1 cleavage site on proIL-33 is not conserved amongst species (Carriere, Roussel et al. 2007). It is also noted that a significant portion of secreted IL-33 is full length proIL-33 (Li, Willingham et al. 2008). Recently IL-33 was shown to be released from macrophages induced to undergo necrotic cell death (Luthi, Cullen et al. 2009). Similar to IL-1 $\alpha$  and HMGB1, IL-33 is released by necrotic cells but kept intracellular during apoptosis, reinforcing a possible role as an ‘alarmin’.

The IL-33/ST2 axis is an attractive therapeutic target given the ability of IL-33 alone to induce asthma like features independent of the adaptive immune system; as demonstrated in this thesis by the increased airway sensitivity shown to IL-33 administration *in vitro*. Studies using recently generated IL-33 KO mouse models have confirmed the role of IL-33 in the innate response for T cell independent allergen induced inflammation and OVA sensitisation (Oboki, Ohno et al. 2010; Oboki, Ohno et al. 2010) and demonstrated that IL-33 contributes to production of T<sub>H</sub>2 associated cytokines and is linked to eosinophil infiltration and histopathologic changes in the lung (Louten, Rankin et al. 2011). Previous studies have shown that administration of IL-33 results in AHR independent of CD4<sup>+</sup> T cells (Kondo, Yoshimoto et al. 2008), shown in RAG-2 KO mice lacking both T and B cells, in which IL-33 administration also induced goblet cell hyperplasia and eosinophilic infiltration. Other studies have shown IL-33-driven activation, degranulation, enhanced adhesion and survival of eosinophils (Cherry, Yoon et al. 2008; Suzukawa, Iikura et al. 2008). IL-33 stimulates other innate immune cells such as basophils for migration (Cherry, Yoon et al. 2008) and production of IL-13, IL-4 and IL-5 in the

absence of allergen or IgE (Smithgall, Comeau et al. 2008; Pecaric-Petkovic, Didichenko et al. 2009). IL-33 promotes maturation, survival, adhesion and cytokine production in mast cells and mast cell progenitors (Ali, Kay et al. 2007; Allakhverdi, Smith et al. 2007; Ho, Ohno et al. 2007; Iikura, Suto et al. 2007; Xu, Jiang et al. 2008). IL-33 administration *in vivo* has also been shown to promote IgE production without infection or allergen present (Schmitz, Owyang et al. 2005).

Further more IL-33 plays a role in amplifying the inflammatory response to such infectious agents as parasites, bacteria and virus which can lead to exacerbation of atopic disorders (Schwarze, Hamelmann et al. 1997; Herz, Lacy et al. 2000; Holtzman, Morton et al. 2002; Lemanske 2004; Marone, Spadaro et al. 2006). IL-33 is produced from epithelial cells in the respiratory tract or gastrointestinal tract that have been activated through pattern recognition receptors which recognise PAMP (previously described) associated with these invading pathogens. IL-33/ST2 signalling plays a role in the local induction of the innate response which can further exacerbate airway inflammation; through increasing levels of eosinophils, lymphocytes, macrophages, IL-13, TGF- $\beta$ , CCL3, CCL17 and CCL24 in the lung (Stolarski, Kurowska-Stolarska et al. 2011). These findings may provide explanation for the allergen independent inflammation seen in non-atopic asthmatics (Smith 2010) and contribute to the diverse phenotypes exhibited in patients with asthma.

This suggests an obvious opportunity for therapeutic intervention, although further studies are required to determine the safest way to interrupt the IL-33/ST2 receptor down stream pathway with out impairing innate immune function; perhaps through targeting of the ST2 receptor. The soluble form of the ST2 receptor acts as a decoy receptor for IL-33 (Oshikawa, Kuroiwa et al. 2001; Hayakawa, Hayakawa et

al. 2007). Previously pretreatment with ST2s in a murine model of asthma resulted in decreased production of IL-4, IL-5, and IL-13 from IL-33 stimulated splenocytes (Hayakawa, Hayakawa et al. 2007). Disruption of IL-33/ST2L signalling has also been shown through use of both an anti-T1/ST2 mAb and recombinant T1/ST2 fusion protein, preventing eosinophil infiltration and  $T_H2$  cytokine secretion (Lohning, Stroehmann et al. 1998).

Incorporating the genetic susceptibility and environmental factors that serve as a predictor for disease risk, recent genome wide association studies aimed at identifying susceptibility signals for asthma have consistently identified both IL-33 (location 9q24) and IL-1RL-1 (the ST2 receptor in human, 2q12) as asthma candidate genes (Kabesch, 2010). The largest and most recent genome wide association study (GWAS) of asthma included ~10,000 case subjects and ~16,000 controls. Strong associations included single nucleotide polymorphisms (SNP) within IL-33 and IL-1RL-1 (Moffatt, Gut et al. 2011). A GWAS had previously identified a SNP at IL-1RL-1 that showed significant association with asthma, prompted by a substudy scanning eosinophil count in blood from > 9000 Icelanders (Gudbjartsson, Bjornsdottir et al. 2009). Additionally, similar significance for association with asthma was observed in 10 different populations (7996 cases, 44890 controls). SNPs within the genes encoding WDR36, MYB and IL-33 also suggested association with eosinophil counts and atopic asthma in these populations. A GWAS investigating SNPs associated with eosinophilia for contribution to susceptibility for nasal polyposis also identified IL-33 (Buysschaert, Grulois et al. 2010). Polymorphisms in IL-1RL-1 have now been associated with asthma and atopy (Reijmerink 2008). Polymorphisms in the IL-1RL-1 gene are also associated with chronic rhinosinusitis (Castano, Bosse et al. 2009) and polymorphisms in the IL-

IL-1RL-1 promoter region occurred more frequently in children with acute asthma, serum levels were also increased in children with acute asthma (Ali, Zhang et al. 2009). Additionally supported by a study showing that polymorphisms in IL-1RL-1 were associated with increased serum soluble IL-1RL-1, levels of blood eosinophils and asthma prevalence in children (Savenije, Kerkhof et al. 2011). In patients with atopic dermatitis a significant genetic association was shown with SNPs in the distal promoter region of IL-1RL-1, regulating expression and influencing activation of the  $T_H2$  response (Shimizu, Matsuda et al. 2005). Interestingly, a study attempting to identify new candidate genes for Alzheimer's disease (AD) found decreased IL-33 expression in brain from AD cases compared to controls; selected polymorphisms within IL-33 were associated with a decrease in AD risk possibly through modulation of cerebral amyloid angiopathy formation (Chapuis, Hot et al. 2009).

These findings taken together with the role of IL-33 in inflammation and AHR demonstrated in this study support IL-33's potential as an 'alarmin' and inflammatory marker of asthma and raise the potential for therapeutic intervention by targeting of the ST2 receptor (IL-1RL-1). Future approaches involve determining the influence of these polymorphisms within IL-33 and the ST2 receptor for initiation, maintenance of the inflammatory response and implications for exacerbation of further disease.

#### **6.4 Animal models of bronchial asthma**

Progression of our understanding of asthma in human is reliant on animal models that reflect the manifested disease state. Diseases bearing any resemblance to

asthma rarely occur naturally in other animals, the closest being allergic syndrome in cats (Padrid, Snook et al. 1995) and heaves in horses (Couetil, Hoffman et al. 2007); forcing a need for sensitised animal models of airway inflammation. In humans asthma diagnosis is reliant on phenotype (given the poorly understood underlying pathology); yet clarifying such a phenotype in animal models is difficult. Revelation of the complex interplay between the innate immune system and adaptive response has also led to disagreement over whether the inflammatory environment or the altered ASM is more influential for severity of the disease; impacting on the design of sensitisation models. Due to knowledge of the genomic sequence, immunological tools available, low cost and short gestation period; mouse models are commonly used to gain understanding of the disease (Taube, Dakhama et al. 2004), with studies contributing to justification for pharmacotherapy. Many variations of the sensitisation protocol exist, including method of administration, length and timings of protocol and the foreign protein used for sensitisation. Ovalbumin (OVA) used in my studies for sensitisation of BALB/c mice, remains the most common antigenic protein to use (Bates, Rincon et al. 2009). However, disagreement with the relevance of OVA to disease initiation in human has lead to use of such natural allergens as house dust mite extract (Fattouh, Pouladi et al. 2005). Another difficulty has been the formation of a chronic protocol in animal models to mimic the chronic disease with features of airway remodelling as in human. Extended exposure to aerosolised OVA is reported to result in tolerance in some mouse strains including BALB/c in which airway inflammation generally peaks at 24 - 72 hours after challenge and resolves within one - two weeks (Yiamouyiannis, Schramm et al. 1999; Kumar, Herbert et al. 2004; Lommatzsch, Julius et al. 2006; Bates, Cojocaru et al. 2008); while long term sensitisation by house dust mite shows greater success in retaining

chronic asthmatic features (Fattouh, Pouladi et al. 2005). A novel approach emerging is the simultaneous exposure to multiple antigens, indicating success in the absence of an adjuvant and against tolerance in chronic protocols (persisting at least three weeks after allergen exposure) (Goplen, Karim et al. 2009). Believed to be due to the effect of multiple allergens on stimulation of p38 MAPK phosphorylation in dendritic cells and resulting maintained T cell activation. Development of such tolerance in mouse models provides a major hurdle for the understanding of maintained inflammation in asthma, particularly given the therapeutic implications for targeting this chronic disease in human.

## **6.5 The Precision Cut Lung Slice technique**

In ASM  $\text{Ca}^{2+}$  plays a central role in contractility, secretion of inflammatory mediators, metabolism, cell differentiation and proliferation (Mahn, Ojo et al. ; Berridge 2008; Gerthoffer 2008; Lipskaia, Hulot et al. 2009); processes reported to be altered in patients with asthma. Previously the role of dysregulated  $\text{Ca}^{2+}$  homeostasis in T cells associated with inflammatory and autoimmune diseases was discussed (section 5.4.1). This project was concerned with the influence of altered  $\text{Ca}^{2+}$  handling on contraction in ASM; assessed using the PCLS technique. Throughout this thesis the PCLS technique was used to measure peripheral airway contraction in mouse and pig, a sensitised mouse model of airway inflammation and KO mouse models and slices exposed to selected pharmacological agents and inflammatory mediators. Key advantages of the lung slice technique include many slices cut from each animal being used for multiple contractile experiments or frozen for RNA extraction and histology; preventing tissue wastage.



Difficulties with this technique include the delicate balance between lung inflation with air and agarose, given the risk for over inflation resulting in tearing or damage to airways. The influence of preparation and disruption to the nervous and circulatory system on airway behaviour in the lung slice has not been directly compared to the behaviour of the distal airways in intact lung via MRI/ quantitative CT assessment; comparisons are complicated by the variation in conditions, agonist and airway generation assessed in previous studies. In my experiments using slices from OVA sensitised lung tissue, results indicated that removal from the inflammatory environment resulted in a decrease in allergen induced AHR after 24 - 48 hours post lung removal. This meant all sensitisation experiments had to be performed within 24 hours, with time constraints restricting repeats and length of protocol. Given the difficulties in acquiring tissue, it is not yet known whether removal from the inflammatory environment results in resolution of AHR in human PCLS from asthmatic patients.

## **6.6 Future Studies**

The ability to study the functional properties of some ion channels and receptors and their influence on contraction in ASM has been limited due to a lack of pharmacological tools. RNA interference (RNAi) technology has become a rapidly expanding area of research to target set transcripts and silence genes (Elbashir, Martinez et al. 2001; Zhou, Tsai et al. 2003; Gurney and Hunter 2005). Short interference RNA (siRNA) provides a tool for characterisation of gene function, identification of signalling pathways and has applications for therapeutic intervention in virally infected cells and as drug targets. siRNA has previously been

applied to cells with success and recently attempted in isolated rat cerebral arteries (Corteling, Brett et al. 2007). An obvious area for future experiments would be to optimise conditions for the transfection of lung slices with siRNA targeting selected candidates playing a role in the  $\text{Ca}^{2+}$  signalling pathway (STIM1, Orai1) to study the effects of this gene silencing on airway contractile behaviour. Further aims include loading of slices with a  $\text{Ca}^{2+}$  sensitive dye (Fluo-4) to image capture and measure ASM  $\text{Ca}^{2+}$  oscillation frequency with exposure to various agents, extracellular  $\text{Ca}^{2+}$  conditions and inflammatory mediators. The studies could be extended to include investigation of the mechanisms regulating cilia beat frequency and its modulation (requiring image capture at higher magnification (630x), >200 time points/second (Bergner and Sanderson 2002)); potentially providing a model to assess the influence of allergen or epithelial damage on cilia beat frequency. Finally, the ultimate goal of this project is to apply the lung slice technique to human lung tissue, with contraction experiments designed for basic pharmacological characterisation and measurement of contraction after allergen exposure (IL-33); to better understand the link between the airway inflammatory response and altered  $\text{Ca}^{2+}$  homeostasis leading to AHR in human.

In conclusion, the studies described in this thesis demonstrate the multiple applications of a novel lung slice technique used to study key signalling pathways leading to ASM contraction and to investigate the key mediators involved in the  $\text{T}_\text{H}2$  inflammatory response resulting in airway inflammation and AHR. This work suggests a critical role for IL-33 as a mediator of airway inflammation and AHR. Future studies are required to gain an understanding of the nuclear function and

release for secretion of IL-33. Discovering the identity of the key downstream cytokines targeted by IL-33/ST2 activation and resulting impact on altered ASM  $\text{Ca}^{2+}$  homeostasis leading to AHR has significant therapeutic implications for the treatment of airway hyper-sensitivity and interruption to the inflammatory response.

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